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Screeners Workshop

Drug Evaluation Branch
Drug Research and Development
Division of Cancer Treatment
National Cancer Institute

Southern Research Institute
Birmingham, Alabama
March 19–20, 1973

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Foreword

Recent progress in the chemotherapy of human cancer has resulted from the discovery of more effective antitumor drugs, the optimization of treatment schedules, the increased effectiveness of supportive care, and the application of concomitant and sequential combination chemotherapy. Since its inception, the National Cancer Institute's drug evaluation program has recognized the potential value to the clinician of preclinical studies with drug combinations. Considering the contribution of the increasingly effective interaction of clinical scientists with laboratory scientists to the historical progression toward improved clinical therapy, and in view of the potential importance of combined modalities of treatment to further improvements in clinical therapy, the Drug Evaluation Branch chose to devote the 1973 "screening" workshop to this topic. The workshop was hosted by the Southern Research Institute, Birmingham, Alabama, and we are indebted to Drs. H. E. Skipper, F. M. Schabel, Jr., W. R. Laster, Jr., and the staff at Southern Research Institute; to B. J. Abbott and the Screening Section, DEB; and especially to the participants for the outstanding success of the meeting. This issue of *Cancer Chemotherapy Reports* Part 2 contains the Proceedings of the workshop which covered recent advances within our contract program with respect to studies of combinations of drugs, drugs plus surgery, drugs plus immunotherapy, and drugs plus radiation. Emphasis was placed on the future of combined modalities of treatment at the clinical and preclinical levels, rationales for the selection of drug combinations for preclinical evaluation, plans for the automatic processing of laboratory results, and the complexities of experimental design and analysis of results inherent to studies of combined therapy.

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Planning Combined Therapy—The Interaction of Experimental and Clinical Studies [1]

Stephen K. Carter [2]

Combined therapy can mean either combination chemotherapy, in which two or more drugs are employed, or it can mean combined modality therapy, in which single-drug or combination chemotherapy is used as an adjuvant to surgical treatment or radiotherapy or both. Viewing combined therapy strictly in the chemotherapeutic sense, it is quite evident that, except for the cures with single drugs in choriocarcinoma and Burkitt's lymphoma, the major successes have been achieved by combination chemotherapy (1).

For nearly 20 years, experimental chemotherapists have been fascinated by approaches with multiple-drug combinations but have not investigated them systematically. Instead, their studies have been conducted on the basis of individual instincts and research interests, or they have followed promising leads uncovered in clinical studies. Unlike single-agent chemotherapy, which developed from predictive evidence of drug efficacy in experimental systems like L1210 leukemia, combination drug regimens have not evolved to clinical use from basic experimental data.

Nevertheless, oncologists have developed some broad guidelines concerning the drugs used in designing combination chemotherapy regimens. There are exceptions of empiricism in many studies reported in the literature, but the main thrust of progress still rests largely on thorough consideration of the following critical factors: (*a*) each drug included should be active when used alone against the neoplasm in question; (*b*) the drugs should have different mechanisms of action; and (*c*) the toxic effects of the drugs should not overlap so that each can be given at or near its maximum tolerated dose (MTD).

The overall progress of chemotherapy has been against the hematologic malignancies, especially acute lymphocytic leukemia and advanced Hodgkin's disease, but it has not been translated to the common solid tumors that are the chief cause of cancer deaths in the United States. The obvious reasons given for this failure are the differing cell kinetics in fast- and slow-growing tumors and the relative accessibility of tumor cells to effective drug concentrations. An additional factor, which is often neglected, is the point in the treatment strategy for a given disease at which chemotherapy is introduced. In this respect, it is significant that the local and regional modalities of surgical treatment and radiotherapy are commonly used in the primary treatment of solid tumors and that chemotherapy is relegated to secondary use after their failure. When this fact is coupled with the knowledge that the therapeutic gains attainable by surgical treatment and radiotherapy seem to have plateaued and that secondary treatment is rarely curative in any tumor, it appears that a combined modality therapy would be advantageous in the primary treatment of solid tumors.

The DCT, NCI, has developed from the former Chemotherapy Program and naturally views combined modality therapy from the standpoint of the chemotherapy involved. In spite of the important clinical advances already made with combination chemotherapy, there is a pressing need to evaluate experimental systems that can provide predictive data that would be of use in the rational design of combination regimens. Rather than attempting

[1] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[2] Cancer Therapy Evaluation (CTE), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

after-the-fact validation and understanding of successes in empirical studies, the correct role of the experimental therapist should be to obtain data in anticipation of clinical needs. If this is to be achieved, the experimentalists and clinicians must work closely together to establish effective interaction of experimental and clinical investigations. The purpose of this paper is to discuss some approaches to this problem and point out some of the potential difficulties.

The DCT is currently evolving a dual approach to combined therapy in solid tumors (fig 1). In disseminated (advanced) disease, the activity of single agents is determined and, from these, optimum combination chemotherapy regimens are designed. The active single agents or combinations are then joined with surgical treatment or radiotherapy or both in a combined modality approach to initial treatment of local (early) disease. This dual approach includes four points where a model experimental system could be useful, three of them involving combined therapy.

The principal consideration in the development of an experimental model is how it is to be put to clinical use (fig 2). Among the several possibilities, probably the most feasible and worthwhile objective within present resources would be a system that could predict the success of combinations of existing agents and modalities. Of course, the validity of the system must be well established before undertaking large-scale testing. Also, testing of every combination would be a practical impossibility and selectivity will be required.

With these points in mind, the first critical question to be answered is whether the experimental systems commonly employed by the DCT will be useful in predicting active drug combinations. Certainly, a massive source of data already exists from studies in the L1210 leukemia system, the experimental tumor that has been so valuable in detecting active new single agents. Assuming that the L1210 system will be a valid model, the problem of identifying the drug combinations to be tested can be attacked by either a disease-oriented or a modality-oriented process of selection (fig 3).

The disease-oriented approach may be taken by choosing the ten (approximately) most active single agents employed against each of the major solid tumor types and hematologic malignancies. For example, table 1 lists nine agents used in the treatment of adenocarcinoma of the breast, the best dose schedule for each, and the two-drug combinations (using any two of the nine drugs) which have been clinically tested in the disease (2). By selecting the most effective agents for each disease in this manner, 29 different drugs exhibit established activity when used alone against one or more of the ten tumors shown in table 2. One of the most striking points in this data is how few of the potentially effective two-drug combinations (which could be devised with nine or ten most-active drugs) have actually been tested in each tumor. Rarely does the number tested exceed 20% of the possibilities and it is much less in most of the diseases. Even if combinations that include drugs with similar mechanisms of action are excluded, which may or may not be a valid step, the gaps are still awesome. These gaps will never be filled with the available clinical resources, and they emphasize the need and the potential usefulness of testing combinations in a model that has some predictive value.

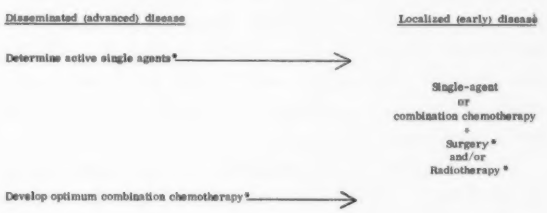

FIGURE 1.—Dual approach to combined therapy in solid tumors; points at which model experimental systems could be used are shown by (*).

To predict a high degree of activity by unknown compounds or combinations (screening)

To predict the success of refinements of therapy with existing compounds or combinations

A. Elucidate principles behind successful clinical therapy

B. Predict successful combinations among the myriad possibilities drawn from active agents and modalities

FIGURE 2.—Clinical uses of an experimental model.

TABLE 1.—Single agents active in breast cancer and clinically tested 2-drug combinations involving these agents

Drug	Schedule	Clinically tested 2-drug combinations
Adriamycin	Single dose, q3wks	Adriamycin + vincristine
Cyclophosphamide	Various schedules, all apparently equivalent	Cyclophosphamide + 5-FU; cyclophosphamide + prednisone
5-FU	Daily × 5 or weekly	5-FU + vincristine; 5-FU + vinblastine; 5-FU + prednisone; 5-FU + melphalan
Methotrexate	Daily, daily × 5, or q6h × 24	Methotrexate + thioTEPA
Vincristine	Weekly	See above combinations
ThioTEPA	Daily × 2–4	
Vinblastine	Weekly	
Prednisone	Daily	
Melphalan	Daily or daily × 4, q3–4wks	

When the two-drug combinations that have been clinically tested in each disease are reviewed, there are 38 combinations for solid tumors (fig 4) and 25 combinations for hematologic malignancies (fig 5). For convenience, these 63 combinations are indicated on a quick-reference chart (table 3). Unfortunately, few of these combinations have been directly compared with their single components in a prospective controlled fashion.

Currently, an analysis is in progress in the CTE using the clinical data available for two-drug combinations. If possible, all of the experimental tumor data will be compiled for the clinically tested combinations so that comparisons and correlations between the two can be made. When this analysis is not possible because of schedule differences or gaps in the data, prospective experimental testing will be initiated to determine whether correlations exist. The results, when completed, will be the subject of a future publication.

In the modality-oriented approach to selecting drug combinations, critical drugs or modalities are chosen and tested systematically in combination with all other available agents. The selection criteria (table 4) which could be used to identify the drugs most likely to be of value in clinical combinations include two factors favoring success with drugs that meet their requirements. These positive factors involve agents of broad-spectrum activity (eg, adriamycin and cyclophosphamide) and those having antitumor activity without marrow toxicity (eg, vincristine and bleomycin). On the negative side, agents with a narrow activity spectrum (eg, mithramycin in testicular tumors and 6-mercaptopurine or cytosine arabinoside against leukemias) would be of low interest. Drugs with slim therapeutic indexes, such as mitomycin C and streptonigrin, also would have low priority. Finally, investigational agents of unproven activity might be avoided since the data would be of minimal clinical value if the drug proved inactive or highly toxic and, thus, of no use to clinicians who are designing combination regimens.

Disease-oriented survey

1. Choose critical tumors
2. Isolate active agents
3. Prospectively test combinations of these agents

Modality-oriented survey

1. Choose critical drugs or modalities
2. Test these systematically in combination with all other available agents

FIGURE 3.—Possible approaches to selection of drug combinations for experimental studies.

TABLE 2.—Single drugs most active in the major tumor types and disease-oriented comparison of potential and clinically tested 2-drug combinations

Tumor types	Most active single drugs	Disease-oriented view of 2-drug combinations using the most active drugs			
		No. of active drugs	Potential combinations	Combinations actually tested	
				No.	Percent
Solid tumors					
Lung	Cyclophosphamide, mechlorethamine, methotrexate, adriamycin, CCNU, methyl-CCNU, BCNU, procarbazine, hexamethylmelamine, hydroxyurea	10	45	8	18
Breast	Adriamycin, cyclophosphamide, 5-FU, methotrexate, vincristine, thioTEPA, vinblastine, prednisone, melphalan	9	36	8	22
Gastrointestinal	5-FU, 5-FUDR, methyl-CCNU, CCNU, BCNU, cyclophosphamide, mitomycin C, methotrexate, adriamycin, streptozotocin	10	45	8	18
Ovary	Melphalan, cyclophosphamide, chlorambucil, 5-FU, methotrexate, actinomycin D, thioTEPA, hexamethylmelamine, adriamycin	9	36	2	6
Melanoma	NSC-45388, BCNU, methyl-CCNU, cyclophosphamide, actinomycin D, hydroxyurea, melphalan, vincristine, CCNU, procarbazine	10	45	10	22
Testicular	Actinomycin D, mithramycin, chlorambucil, vinblastine, melphalan, bleomycin, methotrexate, vincristine, mechlorethamine, adriamycin	10	45	7	15
Hematologic malignancies					
Acute lymphocytic leukemia	Vincristine, prednisone, 6-MP, methotrexate, daunorubicin, L-asparaginase, cyclophosphamide, cytosine arabinoside, adriamycin, BCNU	10	45	8	18
Acute myelocytic leukemia	Cytosine arabinoside, daunorubicin, adriamycin, 6-thioguanine, 6-MP, vincristine, prednisone, cyclophosphamide, BCNU, CCNU	10	45	7	15
Multiple myeloma	Melphalan, cyclophosphamide, prednisone, BCNU, CCNU, vincristine, adriamycin, procarbazine, bleomycin	9	36	6	17
Malignant lymphoma	Cyclophosphamide, mechlorethamine, vinblastine, vincristine, procarbazine, CCNU, BCNU, bleomycin, adriamycin, prednisone	10	45	9	20

Cyclophosphamide + methyl-CCNU *	5-FU + streptozotocin
Cyclophosphamide + streptozotocin	5-FU + BCNU*
Cyclophosphamide + 5-FU	5-FU + CCNU
Cyclophosphamide + methotrexate*	5-FU + methyl-CCNU
Cyclophosphamide + vincristine	5-FU + mitomycin C
Cyclophosphamide + hexamethylmelamine	5-FU + prednisone
Cyclophosphamide + prednisone*	5-FU + melphalan
Cyclophosphamide + NSC-45388	5-FU + vinblastine
Cyclophosphamide + CCNU	5-FU + vincristine
BCNU + mitomycin C	Vincristine + actinomycin D
BCNU + vincristine*	Vincristine + NSC-45388*
BCNU + NSC-45388	Vincristine + adriamycin
CCNU + hydroxyurea	Vincristine + CCNU
CCNU + mechlorethamine	Vincristine + bleomycin *
Vinblastine + bleomycin*	NSC-45388 + actinomycin D
Vinblastine + melphalan	NSC-45388 + procarbazine
Methotrexate + mechlorethamine	NSC-45388 + methyl-CCNU
Methotrexate + hexamethylmelamine	Actinomycin D + chlorambucil
Methotrexate + thioTEPA	Actinomycin D + mechlorethamine

FIGURE 4.—Two-drug combinations clinically evaluated in solid tumors; (*) indicates evidence of synergism.

Prednisone + cyclophosphamide *	Cytosine arabinoside + BCNU
Prednisone + melphalan *	Cytosine arabinoside + CCNU
Prednisone + BCNU *	Cytosine arabinoside + 6-MP
Prednisone + methotrexate *	Cytosine arabinoside + 6-thioguanine *
Prednisone + 6-MP *	Cytosine arabinoside + daunorubicin *
Prednisone + cytosine arabinoside	6-MP + methotrexate
Prednisone + vincristine*	6-MP + Daunorubicin
Prednisone + daunorubicin *	BCNU + cyclophosphamide
Prednisone + adriamycin	BCNU + procarbazine
Prednisone + L-asparaginase	BCNU + melphalan
Vinblastine + vincristine	BCNU + bleomycin
Vinblastine + BCNU	
Cytosine arabinoside + cyclophosphamide *	Adriamycin + bleomycin

FIGURE 5.—Two-drug combinations clinically evaluated in hematologic malignancies; (*) indicates evidence of synergism.

A drug having very broad activity, such as adriamycin (3), could be evaluated in combination with other agents chosen by a disease- and drug-oriented approach. For example, table 5 shows the tumors in which adriamycin is active and lists for each tumor the other active drugs that might be evaluated in combination with adriamycin. Drugs active against more than one tumor are cited only once and are not repeated (eg, cyclophosphamide is active to some degree in all five tumors but is shown only with breast cancer). It is evident that the testing of adriamycin with each of the 22 different drugs active in at least one of the tumors responsive to the antibiotic would cover nearly all of the active clinical agents. When cyclophosphamide is viewed in a similar manner (table 6), the spectrum of drugs is, if anything, even more encyclopedic and emphasizes the need for testing in a model experimental system.

The major difficulties in combining two drugs, either experimentally or clinically, arise in the variety of possible approaches to the variables of dose ratio, schedule, and sequence of drug administration (table 7). In combinations where the drugs do not exhibit overlapping toxicity, ratio is not a problem and both agents can be used at full dose. However, there are three possible solutions when the combined toxic effect of the drugs is a significant problem. One of the drugs can be given at the full dose level while the second is started at a low dose level and escalated to its MTD in the combination regimen. This technique is rarely employed in the clinic but it might be of value when the two drugs are not of equal activity. The most common clinical approach is to use a fixed fraction of the optimum dose for each drug and escalate them together until the MTD is established. Unfortunately, this method may produce a combination of suboptimal doses lacking the anticipated clinical activity.

Table 7 also outlines three possible choices in the sequence of drug administration. Most clinical studies circumvent this problem by administering the two drugs simultaneously. Alternatively, one drug can follow the other in a fixed sequential manner that has been used

TABLE 3.—A quick-reference chart of 2-drug combinations clinically evaluated in solid tumors and hematologic malignancies

Combinations evaluated in solid tumors ↓ / Combinations evaluated in hematologic malignancies →	Mechlorethamine	Cyclophosphamide	Melphalan	Chlorambucil	ThioTEPA	5-FU	Methotrexate	6-MP	6-Thioguanine	Cytosine Arabinoside	Vincristine	Vinblastine	Mitomycin C	Dactinomycin	Adriamycin	Daunorubicin	Bleomycin	BCNU	CCNU	Methyl-CCNU	Streptozotocin	Hexamethylmelamine	Procarbazine	DTIC	Hydroxyurea	L-asparaginase	Prednisone
Mechlorethamine																											
Cyclophosphamide										(H)								H									(H)
Melphalan																		H									(H)
Chlorambucil																											
ThioTEPA																											
5-FU		S	S																								
Methotrexate	S	(S)			S			H																			(H)
6-MP										H						H											(H)
6-Thioguanine										(H)																	
Cytosine Arabinoside																(H)		H	H								H
Vincristine		S				S						H															(H)
Vinblastine			S			S												H									
Mitomycin C						S																					
Dactinomycin	S			S							S																
Adriamycin											S						H										H
Daunorubicin																											(H)
Bleomycin											(S)	(S)						H									
BCNU						(S)					(S)		S										H				(H)
CCNU	S	S				S					S																
Methyl-CCNU		(S)				S																					
Streptozotocin		S				S																					
Hexamethylmelamine		S					S																				
Procarbazine																											
DTIC		S									(S)			S				S		S			S				
Hydroxyurea																			S								
L-asparaginase																											H
Prednisone		(S)				S																					

S = Combination evaluated in one or more solid tumors ((S) = combination showing evidence of possible synergism)

H = Combination evaluated in one or more hematologic malignancies ((H) = combination showing evidence of possible synergism)

in experimental and clinical studies combining drugs of differing cell-cycle specificity (4,5). Three approaches are also available for selecting the treatment schedule, which is perhaps the most complicated of all the variables. The optimum schedule for one of the agents can be used for both agents, or each drug can be given on its optimum schedule even though this may entail non-identical schedules. In the clinical handling of this variable it is surprising to find how often a drug is used in combinations on a schedule that was never

TABLE 4.—Selection criteria for drugs of potential use in combination studies

Positive factors indicating high-priority drugs	Negative factors indicating low-priority drugs
Broad spectrum clinical activity	*Narrow spectrum clinical activity*
Adriamycin	Mithramycin
Nitrosoureas	6-MP
Cyclophosphamide	Hydroxyurea
Methotrexate	Cytosine arabinoside
Lack of clinical marrow toxicity	*Slim therapeutic index*
Vincristine	Mitomycin C
Bleomycin	Streptonigrin
Streptozotocin	Methyl-GAG
Hormonal agents	
	Investigational drugs of unproven clinical value

TABLE 5.—Drugs of potential use in combination with adriamycin based on a disease-oriented view of clinical activity

Tumors against which adriamycin is active	Other drugs active in each tumor
Breast	Cyclophosphamide
	5-FU
	Methotrexate
	Vincristine
	Prednisone
	Testosterone
	Diethylstilbestrol
Sarcoma	Actinomycin D
	NSC-45388
Lung	Mechlorethamine
	BCNU
	CCNU
	Methyl-CCNU
	Hexamethylmelamine
Malignant lymphoma	Bleomycin
	Vinblastine
	Procarbazine
	Streptonigrin
Acute leukemia	6-MP
	6-Thioguanine
	Cytosine arabinoside
	L-Asparaginase

TABLE 6.—Drugs of potential use in combination with cyclophosphamide based on a disease-oriented view of clinical activity

Tumors against which cyclophosphamide is active	Other drugs active in each tumor
Breast	5-FU
	Methotrexate
	Vincristine
	Adriamycin
	Prednisone
	Hormone active drugs
Lung	Mechlorethamine
	Nitrosoureas
	Hexamethylmelamine
Ovary	Melphalan
Malignant lymphoma	Vinblastine
	Procarbazine
	Bleomycin
	NSC-45388
	Streptonigrin
Acute leukemia	6-MP
	6-Thioguanine
	Cytosine arabinoside
	L-Asparaginase
	Daunorubicin
Childhood tumors	Actinomycin D

employed during its evaluation as a single agent.

The three major variables considered in table 7, and the three approaches to each that would yield 27 possible regimens, afford only a minimum view of the complexities inherent in any two-drug combination. Obviously, these 27 combinations, or even more in some situations, can never be clinically evaluated, but valid experimental systems could be the means for selecting the ones of greatest clinical potential.

A final problem in two-drug combination chemotherapy is the evaluation of clinical results, which must be interpreted in terms of

TABLE 7.—Theoretical approaches to problems of dose ratio, sequence of administration, and schedule in 2-drug combination chemotherapy

Variables in 2-drug regimens	Theoretical approaches		
	I	II	III
Dose ratio*	100% Drug A + ×/100 drug B†	×/100 Drug A† + 100% drug B	×/100 Drug A† + ×/100 drug B†
Sequence of administration	Drugs A and B simultaneously	Drug A → drug B	Drug B → drug A
Schedule	Drug A: optimum A schedule Drug B: optimum A schedule	Drug A: optimum B schedule Drug B: optimum B schedule	Drug A: optimum A schedule Drug B: optimum B schedule

*100% = Full dose; ×/100 = fraction of the full dose.
†Escalation to the maximum tolerated dose as in a standard phase I trial.

the antitumor activity that is possible with each drug used alone. Faced with confusing and variously defined terms such as "potentiation," "enhancement," or "synergism," which are used to explain clinically beneficial drug interactions, it is probably best to describe the results simply and represent them numerically as shown in table 8. Here, three types of findings can be considered "positive" in the sense that the activity of the combination is greater than that attained by the optimum use of either drug alone. The superior result is clearly achieved by the combination with activity greater than the additive effect of both agents. On the other hand, the most common clinical observation and the one which is most difficult to interpret is activity that is less than additive but superior to the single agents. In these cases, it is very possible that sequential administration of the individual drugs might equal or surpass the effectiveness of the combination.

Combined therapy, involving either multiple-drug regimens of combination chemotherapy or combined modalities using drugs, surgical treatment, and radiotherapy, has produced almost all of the major triumphs in cancer treatment. There is little doubt that future advances will be made by similar approaches but, hopefully, these will be based on data permitting more rational designs of therapy. It is essential that experimentalists and clinicians work together in evolving the test systems and methods for their use which can move this area forward as rapidly as possible.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127
L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,-3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066

TABLE 8.—Theoretical results with a 2-drug combination

Numerical representation of results	Activity of the combination
2 + 2 = 1	Less active than either single agent alone
2 + 2 = 2	Activity same as either single agent alone
2 + 2 = 3	Activity superior to either single agent alone, but less than additive
2 + 2 = 4	Activity is additive
2 + 2 = 5	Activity superior to additive effects of both agents

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Diethylstilbestrol: NSC-3070; 4,4′-stilbenediol, α,α′-diethyl-; stilbestrol

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

5-FUDR: NSC-27640; 5-fluoro-2′-deoxyuridine

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl] methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Methyl-GAG: NSC-32946; CAS reg. No. 24968-67-0; guanidine, 1,1′-[(methylethanediylidene)dinitrilo]di-, dihydrochloride, monohydrate

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptonigrin: NSC-45383; CAS reg. No. 632-62-2

Streptozotocin: NSC-85998

Testosterone: NSC-9700; CAS reg. No. 58-22-0

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCES

1. CARTER SK. The chemotherapeutic approach to cancer therapy: a quick review. *In* The Year Book of Cancer (Clark RL, and Cumley RW, eds). Chicago, Year Book Medical Publishers, 1972, pp 475–498.
2. CARTER SK. Single and combination nonhormonal chemotherapy in breast cancer. Cancer 30:1543–1555, 1972.
3. CARTER SK, DIMARCO A, GHIONE M, ET AL, eds. International Symposium on Adriamycin. New York, Springer-Verlag, 1972, 251 pp.
4. SCHABEL FM, JR. The use of tumor growth kinetics in planning "curative" chemotherapy of advanced solid tumors. Cancer Res 29:2384–2389, 1969.
5. HANSEN HH, SELAWRY OS, and ANDREWS JR. Treatment of unresectable bronchogenic carcinoma with combination chemotherapy and radiotherapy. Proc Am Assoc Cancer Res 12:80, 1971.

Rationale of Combined Modality Therapy for Cancer [1]

Daniel S. Martin, Ruth Fugmann, Robert Stolfi, and Peggy Hayworth [2]

Multimodal treatment for cancer is not new. Indeed, the use of only a single modality in the treatment of a cancer patient is relatively infrequent. In this regard, the history of the treatment of Wilms' tumor is especially instructive (table 1).

Although the marked improvement in cure rates in recent years is clearly due to the combination of modalities, the use of such combinations has been largely based on empiricism. For example, postoperative radiotherapy to the renal fossa was used only to extend the local area of surgical treatment. Originally, actinomycin D (NSC-3053) was given to patients with advanced or gross clinically evident metastatic disease, but there was no essential change in the survival rate (44%). Hence, the chemotherapy was then administered from the very beginning of surgical treatment and radiotherapy with the hope that it would prevent metastases or tumor masses from arising in possible existing microscopic foci, or might possibly potentiate radiotherapeutic effects. The resultant gratifying leap in cure rates (89%) produced by the employment of the three modalities has been since confirmed by others (139,62,16,20).

More recently, continuing prophylactic maintenance therapy with actinomycin D throughout a 15-month period after surgery, as compared to a single postoperative course of drug, has been shown to be advantageous. The recurrence rate was 14% in the maintenance therapy group, and 52% in the group receiving only a single course; the difference is statistically significant (157).

These results of combined modality therapy demonstrate striking progress in curing patients with no demonstrable metastases on admission. However, the results are disturbingly different if gross metastatic disease is present. Twenty percent to 50% of children with Wilms' tumor, depending on their age and stage of disease at the time of diagnosis, do poorly following surgical treatment plus radiotherapy plus chemotherapy with actinomycin D (147). For example, only a 58% 2-year

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TABLE 1.—History of the treatment of Wilms' tumor*

Period (yrs)	Surgical treatment	Radiotherapy	Chemotherapy	2-Year survivors† (%)
1914–1930	Resection			15
1931–1939	Improved surgery			32
1940–1947	Resection	Postoperative		47
1948–1956	Resection	Postoperative	Actinomycin D for gross metastases	44
1957–1961	Resection	Postoperative	Actinomycin D to prevent metastases	89

*Experience of Boston Children's Hospital (*see* refs 99,30).

†A patient free of disease 2 yrs after treatment of Wilms' tumor may be considered cured; recurrences beyond this time are rare.

survival rate was obtained in patients treated by the three modalities in the presence of demonstrable metastases on admission (30). There is obviously much room for improvement. In the light of recent progress with combination chemotherapy for cancer, it is likely that combination chemotherapy employing actinomycin D and vincristine,[3] an anticancer compound found active against Wilms' tumor, will significantly increase the survival, including cures, in patients with inoperable primary tumors, metastatic disease, and bilateral involvement of the kidneys. Clinical studies of combination chemotherapy after surgical treatment and radiotherapy are in progress.

Clearly, if the explanation for the curative results obtained in the "solid" Wilms' cancer can be placed on a less empirical basis and, rather, be based on sound biologic knowledge, the therapeutic principles discerned should be useful for extrapolation to other solid tumors. This is important. If progress is to be made in the cure of other cancers, as it has in Wilms' tumor, then the precise interaction between the various modalities of cancer therapy needs to be understood. This article summarizes findings from many sources that explain on a scientific basis the curative results obtained in the "solid" Wilms' tumor.

Four fundamental modes of cancer therapy are recognized—surgical treatment, radiotherapy, chemotherapy, and immunotherapy. Of the four, the one that is central to cure, and of pivotal importance to any curative effect of the other therapeutic modalities, is cancer immunotherapy. The practical effectiveness of cancer immunotherapy requires that the host have the ability to develop immunity to cancer. This ability has now been demonstrated. During the past 2 decades, the concept that immunity to cancer can be acquired has been accepted. A large number of excellent review articles summarizing this incontrovertible evidence are available (55,130,152,118,65,48, 66). In short, the existence of tumor-specific antigens, and of an immune response to them, has been well demonstrated in both experimental (47) and human malignant disease including, incidentally, Wilms' tumor (70,25). This proven existence of cancer immunity is not only of academic import; rather, it is the key to cancer prevention, control, and therapeutic cure.

Prophylactically speaking, immunologic factors are believed to be important in the prevention of cancer by acting as host control mechanisms of immunologic surveillance to "search and destroy" the appearance of malignant mutant cells (141,15,14). Indirect evidence indicating that the initial human resistance against cancer is important in vivo is found in the relatively high incidence of tumors that develop in patients receiving immunosuppressive therapy for organ transplants (103), and in the greatly increased frequency of malignancies in patients with immunologic deficiency diseases of inborn errors of the cell-mediated immune apparatus (4). In addition, there has always been supportive indirect evidence for heightened tumor-specific immunologic reactivity in documented cases of spontaneous regression of cancer (29,65).

Aside from its uses in the prophylaxis of cancer, immunity is important in cancer control. Knocking out host immunity with an immunodepressant, such as cortisone, has been found to enhance tumor growth in man (49, 102,26). Also, many studies have intimately linked the capacity of both chemical carcinogens (73,108,136,137) and oncogenic viruses (19,115,119) to achieve carcinogenesis, with their concomitant ability to produce immunosuppression. In addition, the presence of tumors per se is associated with non-specific depression of cell-mediated immunocompetence in the host (132,154,133,138). Thus, there is a good correlation—good, but not entirely reliable as to prognosis (130)—between immunologic reactivity and tumor recurrence after the surgical treatment of cancer. Cancer patients that cannot develop certain cell-mediated immune responses, such as delayed cutaneous hypersensitivity to a skin allergen (eg, 2,4-dinitrochlorobenzene), either have been found to have surgically unresectable

[3] Vincristine: NSC-67574; CAS reg. No. 6449-03-2; leurocristine, sulfate (1:1).

tumors or to have developed early recurrence following definitive surgical treatment of cancer; whereas, in general, patients with normal immunologic competence were found to have a good prognosis (138,28,130).

All of these findings indicating that immunologic mechanisms are important in the control of cancer, naturally suggest that their preservation and strengthening should be capable of producing tumor cure. This curative expectation has been found to be true, but only under circumstances in which the tumor size (or the number of tumor cells) is relatively small, ie, 10^5 cells or fewer (88,89). There is a relationship between the size of a tumor (or the number of tumor cells) and host immunity. In man, for instance, the successful inoculation or "take" of autografts of cancer has been found to depend on the number of malignant cells injected: 10^8 cells always produced a nodule, whereas half that number of logs (10^4) invariably failed to do so, and tumor autograft "takes" employing intermediate cell numbers (10^5–10^7) depended on the state of immunologic resistance of the host (134). Tumor immunity can thus be overcome by a sufficiently large tumor mass (45,64,69). Specifically, there is a critical antigenic mass in the form of a given number of tumor cells beyond which the in vivo immunologic rejection mechanism is ineffective. This discrepancy in the finding of progressive tumor growth in vivo, despite the demonstration of immunologic destruction of tumor cells in vitro, may sometimes be because the adequate immune response needed for tumor eradication in vivo is simply not reached, ie, the host's supply of immune lymphocytes or cytolytic antibodies or both may simply be insufficient to cope with the large number of tumor cells in established clinically evident cancer (159,109). Thus, the number of immune lymphocytes per target number of tumor cells sufficient for tumor eradication in vivo is inadequate when the number of tumor cells, or the size of the tumor, is large. (It appears paradoxical, however, that despite the immunogenicity of tumors, the early "few-cells" stage of tumor growth escapes destruction by host immune mechanisms. It is thought that the first few tumor cells "sneak through" the host's defense mechanisms, and then tumor cell multiplication outruns the supply of host defense cells.)

Another very important mechanism permitting tumors to grow in vivo in spite of an immunity to their tumor antigens is blocking antibodies. These are humoral factors which can abrogate, or block, the destruction of tumor cells by immune lymphocytes, and they are considered a phenomenon akin to efferent immunologic enhancement (54,53,51,50, 60,59,131,104,146,6,7,46). While the nature of the blocking serum factors has not been elucidated, there is suggestive evidence that they may be antigen-antibody complexes, the antigen part of which is provided by the growing tumor (123,124).

Whichever the mechanism, when a tumor has reached the size of 1 billion (10^9) or more cells (only about 1 g of tissue), it is then beyond the capacity of host resistance (1,100,96). Since a neoplasm only 1 cm in size contains approximately 10^9 tumor cells, and patients with widely disseminated neoplastic disease have approximately 1 trillion (10^{12}) cells (36) —which, although a large number, is not much by weight, being merely 2.2 lbs of tumor cells or 1 kg—it would appear that immunotherapy has no therapeutic role for clinically evident cancer. Yet, to the contrary, immunotherapy is actually crucial for the cure of gross clinically evident cancer.

To begin to understand this seeming paradox, it is necessary first to know that the host's ability to express tumor immunity is related to tumor size, being engendered by a small number of growing tumor cells, depressed by a moderate number of tumor cells, and replaced by a state of immunologic nonresponsiveness, or tumor-specific anergy, in the presence of a large number of growing tumor cells (63). Putting it another way, a critical factor in the expression of host immunity is the amount of tumor-specific antigen (ie, the number of tumor cells or the size of the tumor mass) that is present at any time. In the proper amount, tumor-specific antigen will initiate and support an immune response, but when this amount is exceeded, the host's immunologic factors become neutralized.

Thus, the degree of immune depression is related to the quantity of tumor antigen present, namely, the total tumor cell burden of the host. Hence, in the presence of a growing primary tumor, whether or not a small secondary metastatic focus becomes established is correlated with size-dependency on the number of tumor cells already present in the host, as follows: the secondary neoplasm will neither be inhibited nor rejected but will develop in the presence of a *large* primary tumor, it will be inhibited in its growth by a *moderate-sized* primary tumor, and it will be completely rejected after total removal (eg, by surgical, radiotherapeutic, or chemotherapeutic destruction) of the primary tumor (93,67,147,113,9, 142,23,135,58,143). These data clearly reveal that the ability to express tumor immunity is related to the number of tumor cells.

These quantitative relationships—the strength of host immunity versus the number of tumor cells growing in the host—are very important in determining whether or not and to what extent immunity to cancer is expressed, and they help to explain rationally the results obtained with multimodal therapy in Wilms' tumor. Thus, since vascular invasion by Wilms' tumor is very common (34), and thereby distant micrometastases are invariably present, it is still possible for the combined surgical removal-radiotherapeutic destruction of primary Wilms' tumor to result in a 47% cure rate. The explanation is undoubtedly that the surgical removal and radiotherapeutic destruction of the primary Wilms' tumor mass has "released" damped host tumor immunity to overcome the microscopic foci of neoplastic disease. As for the markedly enhanced cure rate (89%), then produced by adding the modality of single agent (actinomycin D) chemotherapy, this result could hardly have ensued without the beneficial concomitant interplay of host immunity, because in Wilms' tumor all elements of the kidney (glandular and stromal) are involved in the malignant transformation. Because of this multielemental composition, a single cell-cycle-specific compound (actinomycin D) would destroy some of the elements, but others would be insensitive. This heterogenicity, ideal for the development or selection of drug resistance (36,87), points to the interaction of concomitant host immunity, "released" by reduction in tumor load due to the surgical-radiotherapeutic-chemotherapeutic treatment. Thus, the complementary effect of this host immunity is undoubtedly the ultimate explanation for the high cure rate (89%) induced by the triple therapy. It is noteworthy, incidentally, that the beneficial effect on host immunity of eradicating the tumor seems to override the potential hazard of using a potent immunosuppressive agent (24) such as actinomycin D. This fact has been well illustrated in animal models (57).

The key principle that emerges from the aforementioned studies is that the number of tumor cells is a critical factor in host antitumor resistance and can decisively influence the ability of the host to resist either progressive localized growth or metastatic dissemination of antigenic tumors. And, of important therapeutic significance, the existing tumor-immune state of the host may be augmented to change quantitative factors in favor of immune defenses over the number of tumor cells present in the host. Such immunotherapy techniques include methods to increase the immunogenicity of tumor cells by tumor vaccines (17,64) or by alterations of tumor cell surfaces using the enzyme, neuraminidase (120, 121,122,10); nonspecific stimulation of host immunity by administration of immunologic adjuvants such as bacillus Calmette-Guérin (BCG) (160,161,75,89,94,95,101,153); the effective transfer of tumor immunity by sensitized lymphoid cells, so-called adoptive transfer of immunity (35,42,33,98,64,100,2,158); and the injection of immune RNA to produce cell-mediated antitumor responses in tumor-bearing animals (2,3,110,111,105,21,106,22). However, all these methods to strengthen host immunity—while admittedly of value—nevertheless are limited in their effectiveness by the size of the established tumor, the tumors being affected only if they are relatively small (ie, < 1 cm or approximately 10^6 cells). The quantitative relationship of tumor size to immunotherapeutic effect is all-important: small

number of tumor cells, good effect; large number, poor effect.

It seems clear that there is a ceiling, and a relatively low ceiling, on the maximal tumor burden approachable by immunotherapy, whether by specific or nonspecific immunotherapy, or by passive adoptive transfer of immune lymphocytes, or by combinations thereof (86).

Despite this sharp limitation of immunotherapy, heed must be paid to immune mechanisms, for they are central to the successful therapy of nonlocalized cancer. Established systemic malignant disease simply cannot be cured without immune mechanisms playing a part. Since chemotherapeutic cure of cancer is dependent on the synergistic interplay of host defenses (85,84,83), the operational interaction of immunity with chemotherapy must be understood if host benefit, namely tumor cure, is to result. Otherwise, the reverse can occur. For example, immunosuppression produced by the prolonged and intensive dosage of anticancer drugs can produce an augmentation of tumor growth and metastatic spread (68,151,112), and reduced therapeutic effects against animal tumors occur if either the anticancer drugs (32,117), or cortisone (82,83), or pre-irradiation (90) severely inhibit host immune response. Conversely, improved therapeutic effects may be achieved by means of minimally or moderately immunosuppressive, but largely tumor-destroying, chemotherapeutic treatments acting in synergism with immunity (91,92,144,83,81,140,148,32, 42,31). However, the latter evidence was obtained with allogeneic animal tumors which, of course, might not be applicable to the autochthonous tumor-host situation. To fully prove the important point that therapeutic cure is indeed dependent on host defenses working synergistically with chemotherapy, it is absolutely necessary that such synergistic therapeutic relationships be demonstrated with autochthonous primary tumors. This has been documented: chemotherapeutic cure has been both augmented by stimulating host immunity with a concomitantly administered nonspecific immunologic adjuvant, and nullified by simultaneous immunosuppression in spontaneous mammary carcinomas growing in their autochthonous murine hosts (82,83,81,79,80,37,78). Further, the combination of chemotherapy with nonspecific immunotherapy has also been found more effective than either mode of therapy alone against spontaneous leukemias (88, 107).

The observed chemotherapeutic effect is inversely correlated with tumor size; namely, the smaller the tumor or the fewer the number of tumor cells, the greater the chemotherapeutic effect. Further, surgery utilized as a tool to reduce the tumor load in the host can increase chemotherapeutic effectiveness (18,86, 76,83,61).

This correlation between optimal therapeutic effect and a low number of target tumor cells is, as previously explained, also the necessary identical condition for effective host immunity, or immunotherapy, to prevail. Thus, as experimentally demonstrated, the biologic link between tumor size and chemotherapeutic response is immunologic, and the biologic interrelationships between tumor size, host defense, and chemotherapeutic response are important synergistic ones affecting cure. Antigen excess as evidenced by the presence of many tumor cells may suppress immune reactivity in the host. Surgical removal of bulk antigenic tumor mass, as in the excision of a large primary Wilms' tumor, can thereby remove a state of immunologic inertia produced by the presence of antigen excess, namely, the large total tumor cell burden in the host (63, 93,67,142,113,135,58,9,74,52).

Another reason why surgical removal of a large primary tumor frequently results in the appearance of increased immunity, may be that the removal of excess tumor antigen may affect the level of blocking antibodies (ie, antigen-antibody complexes) if these are present. Blocking antibodies, which are capable of decreasing or blocking the effect of cell-mediated immunity, tend to decrease or disappear after surgical tumor excision (146,70,6,54,46,52). And it is corroborative of the synergistic therapeutic interrelationship between chemotherapy and host immunity that this therapeutic response is highly correlated with a patient's

immune response prior to treatment (36,43,44, 27); ie, good prognosis to chemotherapy is associated with active immune responses.

Thus, it seems clear from all of the above, that the presence of an active host immunity against a tumor is crucial to the curative therapeutic effectiveness of cancer chemotherapy. However, to achieve cure, chemotherapy must be stopped short of destroying the immunocompetence of the host, and the number of tumor cells left after chemotherapy must be within the ability of the host immunity to destroy. *All* tumor cells must be killed to effect cure. A few viable cancer cells left behind—even just one last cancer cell—can regrow to a lethal tumor (38,155,125,91,87). Recognition of this grim truth has led to the cell-kill hypothesis (125), ie, survival after therapy is quantitatively related to the number of cells killed by treatment, and cure requires killing the last cancer cell.

Unhappily, the ability of cancer chemotherapy *alone* to kill the last cancer cell is not only virtually nil, but the ability of chemotherapy to reduce the tumor load to a level low enough to be overcome by host immunity is severely limited. The reason is that a dose-response relationship exists between the number of cells killed and the dose of an effective drug wherein a particular drug treatment eliminates a constant fraction of the tumor cell mass, not a constant number of tumor cells per dose. The technical term that describes this constant percent reduction in a neoplastic cell population is first-order kinetics (125). This means that a given course of chemotherapy, regardless of the initial number of tumor cells present at the time chemotherapeutic treatment was begun, destroys the same percentage of tumor cells and not the identical absolute number. The fact that cancer chemotherapy obeys first-order kinetics means that the fewer the number of cancer cells present when chemotherapy is initiated, the greater is the chemotherapeutic opportunity for drastically reducing the number of these cancer cells to a very small number, small enough to be eradicated by host immunity.

It has been experimentally verified that the maximal reduction of neoplastic cells by chemotherapy occurs at a time when the initial number of cells being treated is lowest (39, 114,77,125,83,156,126,127,128,18,85,56). Hence, chemotherapy should be most effective after reduction of bulk tumor by either excisional surgical treatment or cell-destroying radiotherapy.

Stated numerically, first-order kinetics means that it takes as much chemotherapeutic treatment to reduce the tumor number from 10^{12} to 10^{9}, as it does for any other three-log reduction. Since host immunity is apparently only effective when the number of tumor cells is low (in the order of 10^{5}–10^{6} cells) and the presence of immunity is essential for the optimal curative effect of chemotherapy, chemotherapy should be, and is, most effective after reduction of bulk tumor by either tumor-removing surgical treatment or tumor-destroying radiotherapy. Hence, after such reduction of bulk tumor load in the host (table 1), the same course of chemotherapy (actinomycin D) that, in the presence of gross Wilms' metastases, yields only a 44% cure rate, doubles this cure rate to 89% when the tumor load is initially still lower, ie, when there are no demonstrable metastases in this latter group of patients as opposed to the former group with gross metastases. The resultant lower number of residual cancer cells after actinomycin D cell-destroying chemotherapy can then fall into the range of effective host immunity, the latter then being able to totally destroy the remaining few tumor cells.

Solid tumors offer a particularly formidable problem for total cell reduction by chemotherapy. First-order kinetics effects on solid tumor cells, as opposed to leukemia cells, are simply not as good. Experimental findings reveal that identical conditions of drug administration resulting in a 99.999% kill of leukemia cells will only reduce solid tumor cells by 90% (129). And in addition to solid tumor

cells being a special problem in resisting optimal chemotherapeutic reduction, theoretical first-order kinetic killing of cancer cells down to the last few cancer cells is not always practically applicable. With most types of drug treatment the chances of killing all the cells by the drug(s) per se decreases gradually as the initially large cell population is reduced to very small numbers (156). And if host immunity, for whatever reason, is not sufficient to mop up the last cancer cell (or cells), the tumor will recur.

Finally, the fact that cancer chemotherapy works by first-order kinetics is not chemotherapy's only limitation. Actually, the major limitation to drug therapy is toxicity. Another way in which the chance to produce chemotherapeutic cure becomes nil, is when the number of tumor cells killed by the drug is more than compensated for by the number of tumor cells proliferating from surviving cells between chemotherapeutic treatments. The available anticancer drugs have a low therapeutic index; there exists only a fine line between therapeutic activity and tolerable toxicity. All anticancer drugs are toxic to the host, and drug toxicity invariably supervenes well before the last cancer cell is killed. An advanced cancer will have a large cancer cell population, and the larger the number of cancer cells to begin with, the greater the number of treatments required to consecutively reduce the cells to the low range required for eliminating the last few cells by host defense mechanisms and the greater the likelihood that prohibitive toxicity will ensue. This toxicity may cause the cessation of chemotherapy prior to the chemotherapeutic reduction of the tumor load to the very low numbers that will be under the ceiling of effective host immunity, thereby ruling out a complete job of total eradication of cancer. This is all the more reason to remove large numbers of tumor cells, as in the Wilms' tumor, by surgical excision and cell-destructive radiotherapy first. Then, the course of chemotherapy given after the surgery-radiotherapy will confront an initial target of tumor cells small enough to permit reduction to a residual number of tumor cells low enough to be approachable for cure by host immunity (eg, the 89% cure rate of Wilms' tumor in table 1).

As regards toxicity, the schedule of chemotherapy can be very helpful in influencing drug effectiveness (40,71,41,149,150,72). Drugs that are relatively ineffective when administered as daily massive therapy, may be quite efficacious if employed on a prolonged but intermittent high-dose schedule. Dose scheduling to maximize tumor damage and minimize host damage can enhance a drug's in vivo preferential toxicity against tumor cells. Also, intermittent chemotherapy has a greater effect on circulating antibody response as compared to continuous treatment which has a relatively greater effect on cellular immunity. Since cellular immunity is the major tumor-restraining facet of the immune response, and since humoral immunity may be enhancing or blocking, intermittent chemotherapy not only provides a lesser overall immunosuppression, but it tends to preserve the important cellular immunity (36).

Toxicity may also be ameliorated by employing combination chemotherapy. Combination of two or more drugs with differing fundamental mechanisms of action can permit a greater effect against a tumor without a corresponding increase in toxicity.

Solid tumors have a markedly heterogeneous neoplastic cell population ideal for the development or selection of drug resistance. Such heterogeneity constitutes a requirement for a multifaceted approach for cure, including combination chemotherapy and immunotherapy (36,87). Further, recognition of the various stages of the cell cycle (8,5,97) and the specificity of drug action in relation to these stages (11,116,12,13,145) has also encouraged the use of combination chemotherapy, and the results have confirmed the attainment of a greater tumor cell kill. Eventually, however, drug toxicity supervenes, usually well before the last cancer cell can be killed by the combination chemotherapy. The only hope for cure, then, is the host defense mechanism, providing it is left sufficiently immunocompetent (weighted toward tumor cell eradication rather than toward blocking immunity), and providing the number of residual tumor cells

is within the ability of host immunity to eliminate.

The cure of cancer requires the eradication of every cancer cell, and in the final analysis only host immunity is capable of destroying all the cells in a cancer population. However, immunity is severely limited by a requirement for a small number of tumor cells. Chemotherapy's limitations (toxicity, immunosuppression, first-order kinetics, and drug resistance) are also severe, but chemotherapy can be markedly tumor-cell-reducing and thereby set the stage for immunity. The obvious limitations to both surgical treatment and radiotherapy are that both are only local treatments and, with few exceptions, cancer is already disseminated when the patient is first diagnosed. All four modalities, then, have severe limitations that preclude cure when used alone. Judiciously combined, however, these modalities can complement each other and can produce the desired result of cure.

It is clear that the documented biologic interrelationships between surgical treatment, radiotherapy, chemotherapy, and immunotherapy require their effective interaction for cure. An approach to neoplastic disease on this overall basis of combined modality therapy, properly employed in combination to utilize complementary advantages, must increasingly yield cancer cures.

REFERENCES

1. ALEXANDER P. Cellular resistance to tumours. Br Med Bull 23:86–92, 1967.
2. ALEXANDER P. Immunotherapy of cancer: experiments with primary tumours and syngeneic tumour grafts. Prog Exp Tumor Res 10:22–71, 1968.
3. ALEXANDER P, DELORME E, HAMILTON L, ET AL. Effect of nucleic acids from immune lymphocytes on rat sarcomata. Nature 213:569–572, 1967.
4. ALEXANDER JW, and GOOD RA. Immunobiology for Surgeons. Philadelphia, W. B. Saunders Co., 1970.
5. ANDREWS JR. Commentary: Combined cancer radiotherapy and chemotherapy: the relevance of cell population kinetics and pharmacodynamics. Cancer Chemother Rep 53:313–324, 1969.
6. BANSAL S, HARGREAVES R, and SJOGREN H. Facilitation of polyoma tumor growth in rats by blocking sera and tumor eluate. Int J Cancer 9:97–108, 1972.
7. BANSAL S, and SJOGREN H. Counteraction of the blocking of cell-mediated tumor immunity by inoculation of unblocking sera and splenectomy: immunotherapeutic effects on primary polyoma tumors in rats. Int J Cancer 9:490–509, 1972.
8. BASERGA R. The relationship of the cell cycle to tumor growth and control of cell division: a review. Cancer Res 25:581–595, 1965.
9. BARSKI G, and YOUN J. Evolution of cell-mediated immunity in mice bearing an antigenic tumor: influence of tumor growth and surgical removal. J Natl Cancer Inst 43:111–120, 1969.
10. BEKESI G, ST. ARNEAULT G, WALTER L, ET AL. Immunogenicity of leukemia L1210 cells after neuraminidase treatment. J Natl Cancer Inst 49:107–118, 1972.
11. BRUCE WR. The action of chemotherapeutic agents at the cellular level and the effects of these agents on hematopoietic and lymphomatous tissue. Can Cancer Conf 7:53–64, 1967.
12. BRUCE WR, MEEKER BE, POWER WE, ET AL. Comparison of the dose- and time-survival curves for normal hematopoietic and lymphoma colony-forming cells exposed to vinblastine, vincristine, arabinosylcytosine, and amethopterin. J Natl Cancer Inst 42:1015–1025, 1969.
13. BRUCE WR, MEEKER BE, and VALERIOTE FA. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered in vivo. J Natl Cancer Inst 37:233–245, 1967.
14. BURNET FM. Immunological Surveiilance. Oxford, Pergamon Press, 1970.
15. BURNET FM. Immunological factors in the process of carcinogenesis. Br Med Bull 20:154–158, 1964.
16. BURGERT EO, JR, and GLIDEWELL O. Dactinomycin in Wilms' tumor. JAMA 199:464–468, 1967.
17. CHECK J, CHILDS T, BRADY L, ET AL. Protection against spontaneous mouse mammary adenocarcinoma by inoculation of heat-treated syngeneic mammary tumor cells. Int J Cancer 7:403–408, 1971.
18. CHIRIGOS MA, COLSKY J, HUMPHREYS SR, ET AL. Evaluation of surgery and chemotherapy in the treatment of mouse mammary adenocarcinoma 755. Cancer Chemother Rep 22:49–53, 1962.
19. CREMER NE, TAYLOR DON, and HAGENS SJ. Antibody formation, latency and leukemia: infection with Moloney virus. J Immunol 99:495, 1966.
20. D'ANGIO GJ. The use of combined actinomycin D and radiotherapy in children with Wilms' tumor. Front Radiat Ther Oncol 4:174–180, 1969.
21. DECKERS P, and PILCH Y. RNA-mediated transfer of tumor immunity—a new model for the immunotherapy of cancer. Cancer 28:1219–1228, 1971.
22. DECKERS P, and PILCH Y. Mediation of immunity to tumor-specific transplantation antigens by RNA inhibition of isograft growth in rats. Cancer Res 32:839–846, 1972.

23. Deckers P, Davis R, and Mannick J. Tumor size and concomitant tumor immunity. Surg Forum 23:112–114, 1972.
24. DeVita VT, Jr, and Carbone P. Chemotherapeutic implications of staging in Hodgkin's disease. Cancer Res 31:1838–1844, 1971.
25. Diehl V, Jereb B, Stjernsward J, et al. Cellular immunity to nephroblastoma. Int J Cancer 7:277–284, 1971.
26. Editorial: Meeting of the Subcommittee on Steroids and Cancer. JAMA 146:655, 1951.
27. Editorial: Immunity in the acute leukemias. Lancet 1:355–356, 1973.
28. Eilber FR, and Morton D. Impaired immunologic reactivity and recurrence following cancer surgery. Cancer 25:362–367, 1970.
29. Everson TC, and Cole WH. Spontaneous Regression of Cancer. Philadelphia, W.B. Saunders Co., 1966.
30. Farber S. Chemotherapy in the treatment of leukemia and Wilms' tumor. JAMA 198:826–836, 1966.
31. Fefer A. Adoptive chemoimmunotherapy of a Moloney lymphoma. Int J Cancer 8:364–373, 1971.
32. Fefer A. Immunotherapy and chemotherapy of Moloney sarcoma virus-induced tumors in mice. Cancer Res 29:2177–2183, 1969.
33. Fefer A. Immunotherapy of primary Moloney sarcoma-virus-induced tumors. Int J Cancer 5: 327–337, 1970.
34. Fernbach DJ, and Martyn DT. Role of dactinomycin in the improved survival of children with Wilms' tumor. JAMA 195:1005–1009, 1966.
35. Fisher J, and Hammond W. Inhibition of tumor growth by syngeneic spleen cell transfer. Surg Forum 17:102, 1969.
36. Frei E, III. Combination cancer therapy: Presidential Address. Cancer Res 32:2593–2607, 1972.
37. Fugmann R, Martin D, Hayworth P, et al. Enhanced cures of spontaneous murine mammary carcinomas with surgery and five-compound combination chemotherapy, and their immunotherapeutic interrelationship. Cancer Res 30:1931–1936, 1970.
38. Furth J, and Kahn M. The transmission of leukemia of mice with a single cell. Am J Cancer 31:276–282, 1937.
39. Goldin A, Venditti JM, Humphreys SR, et al. Influence of the concentration of leukemic inoculum on the effectiveness of treatment. Science 123:840, 1956.
40. Goldin A. Factors pertaining to complete drug-induced remission of tumor in animals and man. Cancer Res 29:2285–2291, 1969.
41. Goldin A, Venditti JM, Humphreys SR, et al. Modification of treatment schedules in management of advanced mouse leukemia with amethopterin. J Natl Cancer Inst 17:203–212, 1956.
42. Glynn J, Halpern B, and Fefer A. An immunotherapeutic system for the treatment of a transplanted Moloney virus-induced lymphoma in mice. Cancer Res 29:515–520, 1969.
43. Gutterman J, Hersh E, McCredie K, et al. Lymphocyte blastogenesis to human leukemia cells and their relationship to serum factors, immunocompetence, and prognosis. Cancer Res 32:2524–2529, 1972.
44. Gutterman J, Rossen R, Butler W, et al. Immunoglobulin on tumor cells and tumor-induced blastogenesis in human acute leukemia. N Engl J Med 288:169–173, 1973.
45. Habel K. The relationship between polyoma virus multiplication, immunological competence, and resistance to tumor challenge in the mouse. Ann NY Acad Sci 101:173–179, 1962.
46. Halliday W, and Miller S. Leukocyte adherence inhibition: a simple test for cell-mediated tumor immunity and serum blocking factors. Int J Cancer 9:477–483, 1972.
47. Hammond WG, Fisher JC, and Rolley RT. Tumor-specific transplantation immunity to spontaneous mouse tumors. Surgery 62:124–133, 1967.
48. Harris J, and Sinkovics J. The immunology of malignant disease. St. Louis, C. V. Mosby Co., 1970.
49. Hartmann WH, and Sherlock P. Gastroduodenal metastases from carcinoma of the breast: an adrenal steroid-induced phenomenon. Cancer 14: 426–431, 1961.
50. Hellström I, and Hellström K. Colony inhibition studies on blocking and nonblocking serum effects on cellular immunity to Moloney sarcomas. Int J Cancer 5:195–201, 1970.
51. Hellström I, Hellström K, Evans C, et al. Serum-mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor-specific antigens. Proc Natl Acad Sci USA 62:362–368, 1969.
52. Heppner G. In vitro studies on cell-mediated immunity following surgery in mice sensitized to syngeneic mammary tumors. Int J Cancer 9:119–125, 1972.
53. Heppner G, and Kopp J. A dilute agar colony inhibition (Cl) test for studying cell-mediated immune responses against tumor cells. Int J Cancer 7:26–33, 1971.
54. Heppner G. Studies on serum-mediated inhibition of cellular immunity to spontaneous mouse mammary tumors. Int J Cancer 4:608–615, 1969.
55. Humphrey L. Tumor immunity. J Surg Res 10:493–512, 1970.
56. Humphreys S, Mantel N, and Goldin A. Chemotherapy and surgery of spontaneous tumors of mice. Eur J Cancer 2:1–7, 1966.
57. Humphreys SR, Chirigos MR, Milstead KL, et al. Studies on the suppression of the homograft response with folic acid antagonists. J Natl Cancer Inst 27:259–276, 1961.

58. JONSSON N, and SJOGREN HO. Specific transplantation immunity in relation to Rous sarcoma virus tumorigenesis in mice. J Exp Med 123:487–503, 1966.
59. KALISS N. Immunological enhancement and inhibition of tumor growth: relationship to various immunological mechanisms. Fed Proc 24:1024–1029, 1965.
60. KALISS N. The elements of immunological enhancement: a consideration of mechanisms. Ann NY Acad Sci 101:64–79, 1962.
61. KARRER K, and HUMPHREYS S. Adjuvant chemotherapy given before or after surgery. Proc Am Assoc Cancer Res 8:35, 1967.
62. KEIDAN S. Actinomycin D in the treatment of cancer in children. Br J Surg 53:614, 1966.
63. KLEIN G, KLEIN E. and CLIFFORD P. Host defenses in leukemia and Burkitt's tumor. Cancer 21:587–594, 1968.
64. KLEIN G, SJOGREN H, KLEIN E, ET AL. Demonstration of resistance against methyl-cholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res 20:1561–1572, 1960.
65. KLEIN G, and OETTGEN H. Immunologic factors involved in the growth of primary tumors in human or animal hosts. Cancer Res 29:1741–1746, 1969.
66. KOLDOVSKY P. Monograph: Tumor specific transplantation antigen. *In* Recent Results in Cancer Research. New York, Springer-Verlag, vol 22, 1969.
67. KOLDOVSKY P, and SVOBODA J. On the question of the mechanism of growth of a tumour against isoimmunity. Folia Biol (Praha) 8:95–99, 1962.
68. KONDO T, and MOORE GE. Production of metastases by treatment with carcinostatic agents. I. Effects of carcinostatic agents on the host. Cancer Res 21:1396–1399, 1961.
69. KRONMAN BS, WEPSIC HT, CHURCHILL WH, JR, ET AL. Immunotherapy of cancer: an experimental model in syngeneic guinea pigs. Science 168:257–259, 1970.
70. KUMAN S, TAYLOR G, STEWARD J, ET AL. Cellular immunity in Wilms' and neuroblastoma. Int J Cancer 10:36–43, 1972.
71. LANE M. Some effects of cyclophosphamide (Cytoxan) on normal mice and mice with L1210 leukemia. J Natl Cancer Inst 23:1347–1357, 1959.
72. LANE M. The effectiveness of cyclophosphamide (Cytoxan) against well established transplanted rodent tumors. Cancer Res 20:1269–1273, 1960.
73. LINDER OEA. Survival of skin homografts in methylcholanthrene-treated mice and mice with spontaneous mammary cancers. Cancer Res 22: 380, 1962.
74. LEFRANCIS D, YOUN J, BELEHRODEK J, ET AL. Evolution of cell-mediated immunity in mice bearing tumors produced by a mammary carcinoma cell line: influence of tumor growth, surgical removal, and treatment with irradiated tumor cells. J Natl Cancer Inst 46:981–987, 1971.
75. LEMONDE P, and CLODE-HYDE M. Influence of bacille Calmette-Guérin infection on polyoma in hamsters and mice. Cancer Res 26:585–589, 1966.
76. MARTIN DS. Experimental design for chemotherapeutic cure of spontaneous mammary mouse carcinoma. Proc Am Assoc Cancer Res 3:248, 1961.
77. MARTIN DS, and FUGMANN RA. A role for chemotherapy as an adjuvant to surgery. Cancer Res 17:1098–1101, 1957.
78. MARTIN DS, and HAYWORTH P. Life-time results of immunotherapy, combination chemotherapy and surgery upon spontaneous breast cancer. Proc Am Assoc Cancer Res 9:45, 1968.
79. MARTIN DS, HAYWORTH P, FUGMANN RA, ET AL. Combination therapy with cyclophosphamide and zymosan on a spontaneous mammary cancer in mice. Cancer Res 24:652–654, 1964.
80. MARTIN D, HAYWORTH P, and FUGMANN R. Enhanced cure of spontaneous murine mammary tumors with surgery, combination chemotherapy, and immunotherapy. Cancer Res 30:709–716, 1970.
81. MARTIN DS. Cancer treatment: immunologic and chemotherapeutic interrelationships. JAMA 178: 723–726, 1961.
82. MARTIN DS. Chemotherapeutic cure of spontaneous mouse cancer. Acta Un Int Cancr 20:185–188, 1964.
83. MARTIN DS, FUGMANN RA, and HAYWORTH P. Surgery, cancer chemotherapy, host defenses and tumor size. J Natl Cancer Inst 29:817–834, 1962.
84. MARTIN DS. Combination versus single drug therapy. Cancer Chemother Rep 10:57–59, 1960.
85. MARTIN DS, and FUGMANN RA. Clinical implications of the interrelationship of tumor size and chemotherapeutic response. Ann Surg 151:97–100, 1960.
86. MARTIN DS. The necessity for combined modalities in cancer therapy. Hosp Prac 8:129–136, 1973.
87. MARTIN DS. Discussion of combination chemotherapy. Ann NY Acad Sci 76:926–929, 1958.
88. MATHÉ G. Immunotherapy in the treatment of acute lymphoid leukemia. Hosp Prac 6:43–51, 1971.
89. MATHÉ G, POUILLARL P, and LAPEYRAQUE F. Active immunotherapy of L1210 leukemia applied after the graft of tumor cells. Br J Cancer 25:814–824, 1969.
90. MIHICH E. Combined effects of chemotherapy and immunity against leukemia L1210 in DBA/2 mice. Cancer Res 29:848–854, 1969.
91. MIHICH E. Modification of tumor regression by immunologic means. Cancer Res 29:2345–2350, 1969.
92. MIHICH E. Host defense mechanisms in the regression of sarcoma 180 in pyridoxine-deficient mice. Cancer Res 22:218–227, 1962.
93. MIKULSKA ZB, SMITH C, and ALEXANDER P. Evidence for an immunological reaction of the host

directed against its own actively growing primary tumor. J Natl Cancer Inst 36:29–35, 1966.
94. Morton D, Eilber F, Malmgren R, et al. Immunological factors which influence response to immunotherapy in malignant melanoma. Surgery 68:158–164, 1970.
95. Morton D, Holmes E, Eilber F, et al. Immunological aspects of neoplasia: a rational basis for immunotherapy. Ann Intern Med 74:587–604, 1971.
96. Morton DL. Cancer immunology and the surgeon. Surgery 67:396–398, 1970.
97. Mueller GC. Biochemical events in the animal cell cycle. Fed Proc 28:1780–1789, 1969.
98. Nadler SH, and Moore GE. Clinical immunologic study of malignant disease: response to tumor transplants and transfer of leukocytes. Ann Surg 164:482–490, 1960.
99. Nealon TF. Management of the patient with cancer. Philadelphia, W.B. Saunders Co., 1965.
100. Old LJ, Boyse EH, Clarke BA, et al. Antigenic properties of chemically induced tumors. Ann NY Acad Sci 101:80–106, 1962.
101. Pearson JW, Pearson GR, Gibson WT, et al. Combined chemoimmunostimulation therapy against murine leukemia. Cancer Res 32: 904–907, 1972.
102. Pelner L. Host-tumor antagonism. XII. Possible effect of the cortisone drugs on the development of metastases in patients with carcinoma. J Am Geriatr Soc 7:428–439, 1959.
103. Penn I, Helgrinson CG, and Staryl TE. De novo malignant tumors in organ transplant recipients. Transplant Proc 3:773–778, 1971.
104. Pierce G. Enhanced growth of primary Moloney virus-induced sarcomas in mice. Int J Cancer 8:22–31, 1971.
105. Pilch Y, and Ramming K. Transfer of tumor immunity with ribonucleic acid. Cancer 26:630–637, 1970.
106. Pilch Y, and Ramming K. Immunological enhancement of murine tumor isografts mediated by RNA from lymphoid organs of xenogenic immunized animals. Transplantation 11:10–19, 1971.
107. Powles R, Kay H, McElwain T, et al. Immunotherapy of acute myeloblastic leukemia in man. In press.
108. Prehn RT. A clonal selection theory of chemical carcinogenesis. J Natl Cancer Inst 32:1, 1964.
109. Priori E, Wilburn J, and Dmochowski L. Immunofluorescence tests on sera of patients with osteogenic sarcoma. J Natl Cancer Inst 46:1299–1308, 1971.
110. Ramming K, and Pilch Y. Mediation of immunity to tumor isografts in mice by heterologous ribonucleic acid. Science 168:492–493, 1970.
111. Ramming K, and Pilch Y. Transfer of tumor-specific immunity with RNA: demonstration by immune cytolysis of tumor cells in vitro. J Natl Cancer Inst 45:543–553, 1970.
112. Reiner J, and Southam C. Increased growth of tumor isotransplants after immunosuppression of the recipient mice by methotrexate or 5-fluoro-2'-deoxyuridine. J Natl Cancer Inst 38:753–759, 1967.
113. Riggins R, and Pilch Y. Immunity to spontaneous and methylcholanthrene-induced tumors in inbred mice. Cancer Res 24:1994, 1964.
114. Roosa R, Weaver CF, and DeLamater ED. Importance of transplant size in chemotherapeutic assay with the use of the Gardner lymphosarcoma. Proc Am Assoc Cancer Res 2:243, 1957.
115. Salomon MH, and Wedderburn N. The immunodepression effect of Friend virus. Immunology 10:445, 1966.
116. Schabel FM. Drug treatment of malignant tumors of man and animals: a rational approach to cancer chemotherapy. South Med Bull 57:40–46, 1969.
117. Schwartz R. Are immunosuppressive drugs self-defeating? Cancer Res 28:1452–1454, 1968.
118. Schwartz R. Immunological aspects of neoplasia. *In* Progress in Experimental Tumor Research. Basel, S. Karger, 1970, vol 13, 180 pp.
119. Siegel BV, and Morton JI. Depressed antibody response in the mouse infected with Rauscher leukemia virus. Immunology 10:559, 1966.
120. Simmons RL, and Rios A. Immunotherapy of cancer: immunospecific rejection of tumors in recipients of neuraminidase-treated tumor cells plus BCG. Science 174:591–593, 1971.
121. Simmons RL, Rios A, and Ray PK. Regression of established methylcholanthrene tumors by intratumor injections of Vbrio cholerae neuraminidase. J Surg Oncol 4:298–305, 1972.
122. Simmons RL, and Rios A. Combined use of BCG and neuraminidase in experimental tumor immunotherapy. Surg Forum 22:99–101, 1971.
123. Sjogren H, Hellström I, Bansal S, et al. Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen-antibody complexes. Proc Natl Acad Sci USA 68: 1372–1375, 1971.
124. Sjogren H, Hellström I, Bansal S, et al. Elution of blocking factors from human tumors, capable of abrogating tumor-cell destruction by specifically immune lymphocytes. Int J Cancer 9:275–283, 1972.
125. Skipper HE, Schabel FM, Jr, and Wilcox WS. Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with "curability" of experimental leukemia. Cancer Chemother Rep 35:1–111, 1964.
126. Skipper HE, Schabel FM, Jr, and Wilcox WS. Experimental evaluation of potential anticancer agents. XIV. Further study of certain basic concepts underlying chemotherapy of leukemia. Cancer Chemother Rep 45:5–28, 1965.
127. Skipper HE. Criteria associated with destruction of leukemia and solid tumor cells in animals. Cancer Res 27:2636–2645, 1967.
128. Skipper HE. Biochemical, biological, pharmacolo-

gic, toxicologic, kinetic and clinical (subhuman and human) relationships. Cancer 21:600–610, 1968.

129. SKIPPER H. Cancer chemotherapy is many things. Cancer Res 31:1173–1180, 1971.
130. SMITH RT. Possibilities and problems of immunologic intervention in cancer. N Engl J Med 287: 439–450, 1972.
131. SNELL G. Immunologic enhancement. Surg Gynecol Obstet 130:1109–1119, 1970.
132. SOLOWEY A, and RAPPAPORT F. Immunologic responses in cancer patients. Surg Gynecol Obstet 121:756–760, 1965.
133. SOUTHAM C. The immunologic status of patients with non-lymphomatous cancer. Cancer Res 28: 1433–1440, 1968.
134. SOUTHAM CM, and BRUNSCHWIG A. Quantitative studies of autotransplantation of human cancer: a preliminary report. Cancer 14:971–978, 1961.
135. STJERNSWARD J. Immune status of the primary host toward its own methylcholanthrene-induced sarcomas. J Natl Cancer Inst 40:13–22, 1968.
136. STJERNSWARD J. Immunodepressive effect of 3-methylcholanthrene: antibody formation at the cellular level and reaction against weak antigenic homografts. J Natl Cancer Inst 35:885–892, 1965.
137. STJERNSWARD J. Effect of noncarcinogenic and carcinogenic hydrocarbons on antibody-forming cells measured at the cellular level in vitro. J Natl Cancer Inst 36:1189, 1966.
138. SUTHERLAND RM, INCH WR, and MCCREDIE JA. Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer. Cancer 27:574–578, 1971.
139. SUTOW WW. Chemotherapy in childhood cancer (except leukemia). Cancer 18:1585–1589, 1965.
140. TARNOWSKI GS, and STOCK CC. Effects of combinations of azaserine and of 6-diazo-5-oxo-R-norleucine with purine analogs and other antimetabolites on the growth of two mouse mammary carcinomas. Cancer Res 17:1033–1039, 1957.
141. THOMAS L. *In* Cellular and Humoral Aspects of the Hypersensitive State (Lawrence HS, ed). New York, Hoeber, 1959, p 529.
142. VAAGE J. Concomitant immunity and specific depression of immunity by residual or reinjected syngeneic tumor tissue. Cancer Res 31:1655–1662, 1971.
143. VAAGE J. Specific desensitization of resistance against a syngeneic methylcholanthrene-induced sarcoma in C_3Hf mice. Cancer Res 32:193–199, 1972.
144. VADLAMUDI S, PADARATHSINGH M, BONMASSAR E, ET AL. Effect of combination treatment with cyclophosphamide and isogeneic or allogeneic spleen and bone marrow cells in leukemia cells in leukemic (L1210) mice. Int J Cancer 7:160–166, 1971.
145. VALERIOTE FA, BRUCE WR, and MEEKER BE. Synergistic action of cyclophosphamide and 1,3-bis-(2-chloroethyl)-1-nitrosourea on a transplanted murine lymphoma. J Natl Cancer Inst 40:935–944, 1968.
146. VANKY F, STJERNSWARD J, KLEIN G, ET AL. Serum-mediated inhibition of lymphocyte stimulation by autochthonous human tumors. J Natl Cancer Inst 47:95–103, 1971.
147. VANWIJCK R, GODRICK E, SMITH H, ET AL. Stimulation or suppression of metastases with graded doses of tumor cells. Cancer Res 31:1559–1563, 1971.
148. VENDITTI JM, and GOLDIN A. Drug synergism in antineoplastic chemotherapy. *In* Advances in Chemotherapy (Goldin A, and Hawking F, eds). New York, Academic Press, 1964, vol 1, pp 397–498.
149. VENDITTI JM, GOLDIN A, and KLINE I. The influence of treatment schedule on the chemotherapy of advanced leukemia L1210 in mice. Cancer Chemother Rep 6:55–57, 1960.
150. VENDITTI JM. Treatment schedule dependency of experimentally active antileukemic (L1210) drugs. Cancer Chemother Rep Part 3, vol 2:35–59, 1971.
151. WATANABE T, and TANAKA K. Effect of carcinostatic agents on carcinomatous metastases in the spleen. Gann 60:611–616, 1969.
152. WEISS D. Immunological Parameters of Host-Tumor Relationships. New York, Academic Press, 1971.
153. WHITE RG. Role of adjuvants in the production of delayed hypersensitivity. Br Med Bull 23:39–45, 1967.
154. WHITTAKER MG, REES K, and CLARK CG. Reduced lymphocyte transformation in breast cancer. Lancet 1(7705):892–893, 1971.
155. WILCOX WS, SCHABEL FM, and SKIPPER HE. Experimental evaluation of potential anticancer agents. XV. On the relative rates of growth and host kill of "single" leukemia cells that survive in vivo Cytoxan therapy. Cancer Res 26:1009–1014, 1966.
156. WILCOX WS. Letter: The last surviving cancer cell: the chances of killing it. Cancer Chemother Rep 50:541–542, 1966.
157. WOLFF JA, KRIVIT W, NEWTON WA, JR, ET AL. Single versus multiple dose dactinomycin therapy of Wilms's tumor. N Engl J Med 279:290–294, 1968.
158. WOODRUFF MFA. Immunological aspects of cancer. Lancet 2:265–270, 1964.
159. WOODRUFF M. Residual cancer. *In* The Harvey Lectures. New York, Academic Press, 1972.
160. ZBAR B, and TANAKA T. Immunotherapy of cancer: regression of tumors after intralesional injection of living *Mycobacterium bovis*. Science 172:271–273, 1971.
161. ZBAR B, BERNSTEIN I, BARTLETT G, ET AL. Immunotherapy of cancer: regression of intradermal tumors and prevention of growth of lymph node metastases after intralesional injection of living *Mycobacterium bovis*. J Natl Cancer Inst 49:119–130, 1972.

Immunologic and Chemotherapeutic Parameters in a Model Breast Tumor System

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The concepts presented in the preceding paper (1) underline the importance of combining therapeutic modalities to allow host immunity to exert maximal antitumor activity. In this context of antitumor immunity, any laboratory tumor model to be utilized for therapeutic studies should relate to the spontaneous human situation. Consequently, it is essential to use as a model a spontaneous experimental tumor in order to properly study the subtle immune interrelationships in the host of origin (the autochthonous host). Of those experimental spontaneous tumor-host systems available, the one which appears to be by far the most clinically relevant model is the mammary tumor virus-induced mouse mammary adenocarcinoma because of its remarkable resemblance to the human breast cancer situation. Many similarities have been noted in regard to anatomic morphology and spreading (2–7), growth and progression through a precancerous stage (6–14), genetic factors (6,11, 15–17), hormonal factors (5,6,11,18,19), and even occasional spontaneous regressions (4,12,20–22).

The use of spontaneous mouse mammary adenocarcinoma as a model for clinical breast cancer has been further fortified by recent findings, some of which have been summarized by Dmochowski et al (23). In murine and human mammary cancer, a viral factor seems to be involved in the etiology, and virus particles found in the milk of mice and women bearing breast tumors are remarkably similar. Sera from patients with breast cancer neutralize the mouse mammary tumor virus, whereas control sera do not. Primary tumors in both women and mice develop in more than one breast and a higher breast cancer incidence is evidenced within family groups of both.

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Moore and his collaborators (24,25) have found a human subpopulation (Parsi women in Bombay) whose high incidence of breast cancer, as compared with the rest of the population of Bombay, bears some resemblance to the high incidence found in certain strains of inbred mice. The slow-growing characteristics of the tumor model, as well as the general correlation of response of human cancer and of this murine tumor to certain chemotherapeutic agents, as noted by the group at Southern Research Institute (personal communication), by Sandberg and Goldin (26), by Di Marco et al (27), and by ourselves (28, 29, and unpublished results), further underscore their resemblance.

Considering the importance of immune mechanisms in determining the ultimate course of neoplastic disease, as well as the proven value of surgical treatment and the growing potential of chemotherapy for that disease, we have deemed it essential to elucidate the state of immunity in a tumor-host undergoing surgical treatment plus combination chemotherapy. We wish to determine whether the immune status of the autochthonous host during treatment may be used as a guideline (*a*) to better schedule, or select, the chemotherapy so as to increase tumor cures without host toxicity, and (*b*) to better schedule, or select, any immunotherapy to be incorporated into the regimen.

For this purpose, we have developed "baseline" therapy that consists of surgical treatment plus combination chemotherapy which is partially effective at the level of lifetime spontaneous tumor cures. The therapy is effective enough to measure the contribution of host immunity toward producing those cures and to evaluate the antitumor effects resulting from alterations in administration, or in content, of that chemotherapy as suggested by the immunologic findings.

This paper describes our tumor-host system, the baseline therapy, and some of the effects of one course of that therapy on the humoral and cellular immune responses of the host.

The Tumor-Host System

A large colony of $CD8F_1$ female mice (30), which shows a high incidence of mammary tumors as a result of natural neonatal infection with the mammary tumor virus (via the mother's milk), is maintained at our institution. The animals are a first generation hybrid resulting from a cross of Balb/c females and DBA/8 males, both of which carry the tumor-inducing virus. The cross was developed to produce a spontaneous tumor-bearing mouse of sufficient hardiness to withstand experimental therapeutic procedures including anesthesia, surgery, and multicompound chemotherapy. The $CD8F_1$ females develop mammary tumors at an average age of 10 months and at an incidence of 75%–80%.

Mammary tumors of this type have been shown to contain a common virus-associated transplantation antigen(s), as well as individual tumor-specific neoantigens which appear to be antigenically unrelated to the virus.

The spontaneous appearance of tumors in these animals is largely a result of their state of at least partial immune tolerance to the tumor-inducing virus, presumably elicited as a result of neonatal infection prior to the development of complete immunologic competence. Consequently, the mouse appears unable to mount a good immune response against the common, virus-associated tumor antigen(s). However, the animal can, and does, respond immunologically to the individual tumor-specific antigen(s) when it appears in conjunction with tumor development later in life. Nevertheless, few (if any) tumors are spontaneously rejected, and untreated mice succumb to the tumor within 1–2 months of its gross detection.

Baseline Therapy

Animals bearing single spontaneous tumors first undergo surgical treatment to remove the bulk of the tumor. The tumor is stripped out grossly, deliberately leaving behind microscopic foci of tumor cells. The animals are then separated by random distribution into groups of 20–30 mice for further treatment.

Chemotherapy consists of a combination of four agents: 6-thioguanine (8 mg/kg); cyclophosphamide (20 mg/kg); actinomycin D (20 μg/kg); and mitomycin C (1 mg/kg).[2] (The acronym, TEAM, has been devised to refer to this combination.)

This combination of four agents has been administered as follows: four weekly injections beginning the day after surgical treatment; a 2-week rest; four weekly injections; a 4-week rest; four weekly injections. The schedule represents a total of 12 injections over 105 days. Any animal dying within 30 days after the last injection of chemotherapy (ie, within 135 days after the original surgical treatment) is recorded as a therapy death. Prior to 135 days, only animals dying with tumors are considered in determining the cure rate. The absence of a recurrence of the primary tumor in the period between 135 days after surgical treatment until the death of the animal is scored as a local cure; the absence of a recurrence of the original tumor and the development of no new tumor in the same period of time is scored as an absolute cure.

Our total experience with this regimen of TEAM will be presented elsewhere. The local and absolute cure rates, however, in a typical experiment utilizing the baseline therapy are shown in table 1. It can be seen that the addition of TEAM to surgical treatment increases the local cure rate by 37% and the absolute cure rate by 16%.

Our overall results have shown that the increase in local and absolute cure rates produced by TEAM is highly statistically significant and we consider these rates to be at a level which will allow the effects of any alterations in the treatment to be easily detectable in terms of cure rate and of the general well-being of the host.

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[2] 6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan; endoxan.

Actinomycin D: NSC-3053.

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7.

TABLE 1.—Effect of surgical treatment and TEAM* on cure rate of spontaneous breast tumors in autochthonous hosts

Expt No.	Treatment group (20 mice/group)	Local cures† (%)	Absolute cures‡ (%)	Drug deaths (%)
897	Surgical treatment§	5	0	0
	Surgical treatment + TEAM	42	16	10

*TEAM = 6-thioguanine (8 mg/kg); cyclophosphamide (20 mg/kg); actinomycin D (20 μg/kg); mitomycin C (1 mg/kg). This combination of agents was administered as follows: 4 weekly injections beginning the day after surgical tumor enucleation; 2 weeks' rest; 4 weekly injections; 4 weeks' rest; 4 weekly injections, for a total of 12 injections over a 105-day period following surgery.

†Local cure = no recurrence of enucleated primary tumor.

‡Absolute cure = no recurrence and no new tumor at another breast site; any animal dying without a tumor before 30 days after the last injection (ie, 135 days after surgery) is considered a therapy death and is not utilized in determining cure rate.

§Surgical treatment performed at approximately 10 months of age, the average time of tumor appearance. Removal was enucleative (as opposed to excisional), so as to leave microscopic foci of tumor cells locally.

Investigations into some of the immunologic effects of the baseline therapy have been conducted. Initially, data were accumulated from animals that received only a single series of four weekly injections of chemotherapy (ie, one third of the total regimen). These experiments were done in male mice first rather than in female mice to insure the absence of tumors, either initially present or appearing during the test period, that could unnecessarily complicate the picture at this phase of investigation. Experiments utilizing tumor-bearing male animals were done with first generation tumor transplants so that all tumors within an experiment would share the tumor-specific antigen(s)and, as a result, all animals would have an immune response specific for a particular antigenic complement.

Effects of Baseline Therapy on Blood Elements and Bone Marrow

Utilizing nontumor-bearing male mice that received a single course of TEAM, we first examined the effects of the chemotherapy on the number of red and white blood cells in the peripheral blood. Figure 1 shows the results of the four weekly injections of TEAM (a single course) on the blood cell counts. It can be seen that a progressive anemia and a progressive leukopenia developed from which the animals recovered in about 3–4 weeks. This was the first bit of evidence that suggested that a rest period of only 2 weeks before be-

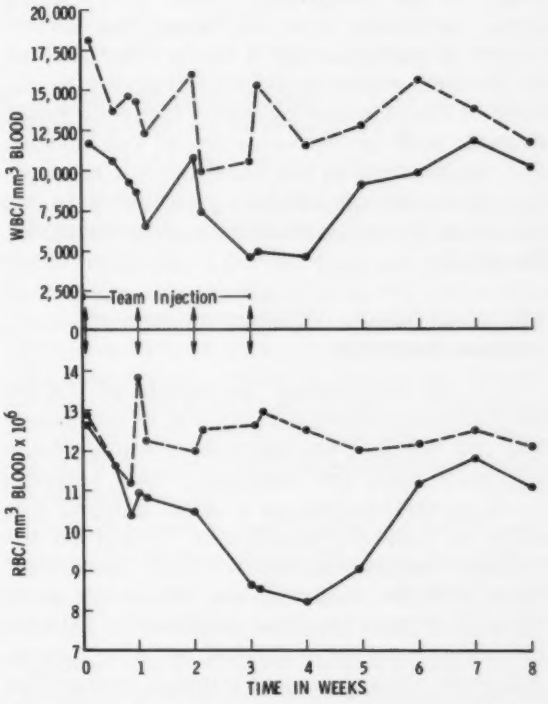


FIGURE 1.—Effect of TEAM on peripheral blood cell counts of nontumor-bearing CD8F$_1$ male mice (average of five samples per point). Control = -----; TEAM = ————.

ginning the second course of chemotherapy may not be sufficient for the animals to recover.

We also examined the bone marrow of animals that received one course of the chemotherapy. The animals were killed and smears of their bone marrow were made on the day after the last of the four weekly injections as well as 1 month after the fourth weekly injection. The results showed that the leukopenia noted in the peripheral blood was primarily a lymphopenia. The percentage of lymphocytes in control animals was 57% of the total cells counted, whereas it was only 18% of the total cells counted in the TEAM-treated mice. This effect, noted the day after the fourth injection of TEAM, had completely disappeared by 1 month after the chemotherapy.

The actual time of bone marrow recovery within that 4-week period has not yet been determined, but it is probably shortly before the return to normal of the white blood cell count in the peripheral blood. This finding again suggested that initiating the second course of chemotherapy 2 weeks after the end of the first course could be dangerous to the host, in that the second course probably would coincide with an active period of cell division and replacement in the bone marrow and render those cells particularly susceptible to the effects of the cell-cycle-specific chemotherapeutic agents.

Effects of Baseline Therapy on Humoral Immune Response

We next determined the effects of TEAM on the humoral immune response of the mouse. For this purpose, we also used nontumor-bearing male mice and measured their response to sheep erythrocytes as a model antigen. The effect of a single injection of TEAM on the primary immune response to sheep E was measured, and the response was shown to vary, depending upon the time relationship between the administration of TEAM and the antigen. Although some degree of immune suppression usually resulted, there was no suppression of the antibody response to 1×10^7 sheep erythrocytes when TEAM and the cells were given simultaneously (fig 2). In fact, a slight enhancement in response consistently occurred. However, when the sheep erythrocytes were administered simultaneously with only the second, third, or fourth injection of TEAM, a gradual increasing inhibition of the primary immune response was noted. The effects of giving sheep cells with the fourth injection of TEAM, or on the first, second, or third week after the fourth injection of TEAM, are shown in figure 3. The inhibition of the primary immune response to the antigen can be seen to persist for up to the third week after the single course of combination chemotherapy. These data measuring antibody response indicated that a rest period of 2 weeks between the first and second courses of chemotherapy was too short for complete recovery of the immune capacity of the host.

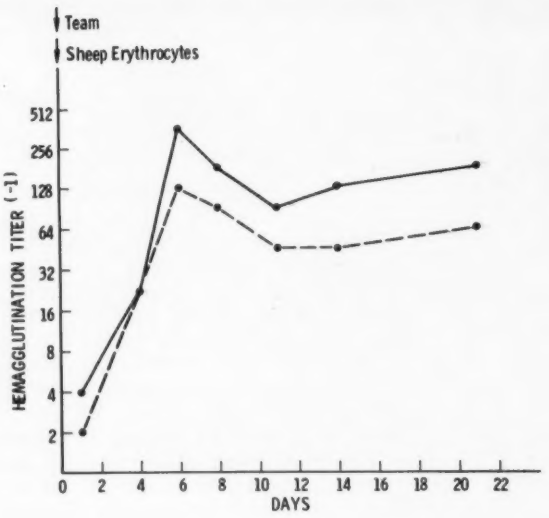


FIGURE 2.—Effect of simultaneous administration of TEAM and 1×10^7 sheep erythrocytes on primary antibody response in nontumor-bearing $CD8F_1$ male mice. Control = - - - - -; TEAM = ————.

Of course, this type of experiment was designed to measure the primary immune response of the mouse to an antigen never seen before (ie, a situation similar to one in which an animal might initially respond to a vaccine of altered tumor cells given as immunotherapy or to a new tumor appearing during the course of therapy). In order to measure the effect of the chemotherapy on the anti-

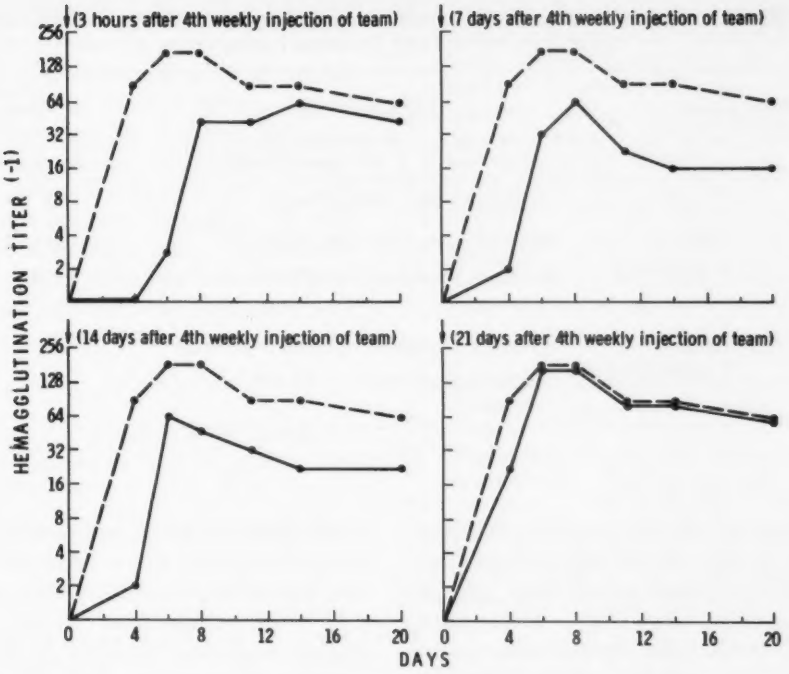


FIGURE 3.—Effect of four injections of TEAM on primary antibody response of non-tumor-bearing CD8F$_1$ male mice: 1×10^7 sheep erythrocytes (↓). Control = - - - - -; TEAM = ————.

body response to an antigen present throughout the treatment (ie, a situation analogous to residual tumor after surgical failure or to a metastasis), we are presently giving sheep cells simultaneously with every injection of TEAM. The findings to date suggest that the antibody response is less suppressed, if at all, to an antigen constantly present during TEAM therapy.

Effects of Baseline Therapy on Cellular Immune Response

The last aspect of these immune studies to be presented concerns the effect of TEAM on the cell-mediated immunity of an animal as expressed against its own tumor cells. These experiments were done in male mice bearing first generation tumor transplants so that a sequential study could be done with pooled materials from treated and control animals bearing tumors of the same antigenic specificity. The experiments consisted of an in vitro assay of the lymph node cells of an animal (or animals) for cytotoxic action against its tumor cells (31). Cytotoxicity was measured by counting the number of cultured tumor cells remaining after incubation with potentially immune versus normal lymphocytes. A statistically significant reduction in the number of residual tumor cells after exposure to immune as compared to control lymphocytes was considered to indicate active cell-mediated immunity against the tumor.

Table 2 shows some of the data of my colleague, Dr. Robert Stolfi. The results of two experiments are presented in which tumor-bearing male mice received strip surgical treatment alone and surgical treatment plus TEAM. It can be seen that untreated tumor-bearing animals sometimes did and sometimes did not evidence antitumor immunity. (Dr. Stolfi's more recent experiments show that the presence or absence of cellular immunity in the tumor-bearing animals bears a relation to the

TABLE 2.—Effect of surgical treatment and surgical treatment plus TEAM on lymphocyte cytotoxicity for target tumor cells

No. of days after—		Lymphocyte donors (5×10^5 lymphocytes/well of 2×10^5 target cells)	Reduction of target cell No. (%)	
Surgical treatment	TEAM		Expt 1	Expt 2
0	0	Tumor-bearing (presurgery)	17	31*
8	7 (after 1 injection)	Surgical treatment (no tumor)	21*	32†
		Surgical treatment + TEAM × 1 (no tumor)	30†	28†
29	7 (after 4 injections)	Surgical treatment (tumor regrowth)	–8	—
		Surgical treatment + TEAM × 4 (no tumor)	47†	—

*$P = 0.05$.
†$P = 0.001$.

size of their tumors.) Within a week after surgical removal of the tumor, all animals evidenced immunity whether or not they had received one injection of TEAM. Even more significantly, 1 week after four weekly injections of TEAM (one course), cell-mediated immunity was not measurably suppressed and the surgical treatment plus TEAM group of mice still had active, immune lymphocytes, whereas the lymphocytes from animals that received only surgical treatment and in which the tumor had regrown no longer showed cytotoxicity for the target tumor cells.

The data clearly show the effectiveness of surgically removing a large tumor in releasing cell-mediated immunity in an animal. Further, the results show that the one course of TEAM did not suppress the cellular immunity to any detectable degree. (Since this type of experiment is not quantitative in nature, it cannot presently be ascertained whether the cell-mediated antitumor immunity may be partially inhibited.)

One final observation concerns the "blocking factor," or "blocking antibodies," found in the sera of many tumor-bearing animals. Dr. Stolfi is currently studying this factor by examining pools of sera from the same groups of mice that provided the lymphocytes for the cytotoxicity tests. The presence of tumor-enhancing factor in the serum is determined by exposing the target tumor cells in culture to the serum, removing the serum, and adding known cytotoxic lymphocytes. Significant reversal of target cell cytotoxicity after exposure to the serum (ie, more target cells remaining than in the test with lymphocytes alone) documents the presence of the blocking factor. Preliminary findings have shown that the blocking factor was present in the sera of all tumor-bearing mice examined. In addition, it became undetectable 2 weeks after surgical treatment but reappeared as soon as the tumor began to regrow in the animal. In the case of the enhancing factor, no difference between mice receiving surgical treatment alone or surgical treatment plus TEAM has yet been seen. More precisely, TEAM does not appear to inhibit or suppress the reappearance of the enhancing factor in the face of tumor recurrence (unpublished observation).

Our present findings are interpreted to show that: (*a*) combined modality therapy, consisting of surgical tumor enucleation plus multicompound chemotherapy, is effective in producing significant numbers of lifespan tumor cures in the autochthonous host; (*b*) tumor removal, in this case by surgical treatment, serves both to release cell-mediated immunity in the host and to effect the disappearance of the blocking factor from the serum; (*c*) the addition of one course of TEAM to the surgical treatment does not abrogate cell-mediated immunity (on the other hand, it does not appear to inhibit the reappearance of the enhancing factor when the tumor recurs); (*d*) one course of TEAM has

adverse effects on the bone marrow, peripheral blood, and the primary antibody response from which the animals recover in about 3–4 weeks. (These findings suggest that a period longer than 2 weeks might best be observed between the first and second courses of chemotherapy in terms of host toxicity and in terms of host immunity against a new tumor appearing during therapy); and (*e*) immunotherapy (specific or nonspecific) might best be administered simultaneously with at least the first, and possibly other, injections of TEAM.

REFERENCES

1. MARTIN DS, FUGMANN R, STOLFI R, ET AL. Rationale of combined modality therapy for cancer. Cancer Chemother Rep Part 2, vol 4:13–24, 1974.
2. WEISS DW. Discussion. *In* Specific Tumor Antigens (Harris RJC, ed). Copenhagen, Munksgaard, 1967, p 186.
3. CONSOLANDI V, VERONESI U, and BRIZIARELLI G. Metastatic spread of mammary cancer: a comparative study in human beings and in mice. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 791–804.
4. DMOCHOWSKI L. The importance of studies on mammary tumor-inducing virus in the problem of breast cancer. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 655–708.
5. MUHLBOCK O. Mammary cancer in human beings and in animals. A comparison. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 811–816.
6. SEVERI L, and SQUARTINI F. A parallel study of human and experimental breast cancer. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 835–845.
7. BONSER GM, DOSSETT JA, and JULL JW. Human and Experimental Breast Cancer. Springfield, Ill, C. C. Thomas, 1961.
8. BONSER GM. A microscopical study of the evolution of mouse mammary cancer: the effect of the milk factor and a comparison with the human disease. J Pathol Bact 57:413–422, 1945.
9. BERN HA, DEOME K, ALFERT M, ET AL. Morphologic and physiologic characterization of hyperplastic nodules in the mammary gland of the C3H/He CRGL mouse. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 565–573.
10. FOULDS L. The development of mammary tumors. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 501–503.
11. BERN HA, and NANDI S. Recent studies of the hormonal influence in mouse mammary tumorigenesis. Prog Exp Tumor Res 2:99–114, 1961.
12. SQUARTINI F. Responsiveness and progression of mammary tumors in high-cancer-strain mice. J Natl Cancer Inst 28:911–926, 1962.
13. DEOME K, and NANDI S. The mammary tumor system in mice, a brief review. *In* Viruses Inducing Cancer (Burdette WJ, ed). Salt Lake City, University of Utah Press, 1966, pp 127–137.
14. LIEBETT AG, and LIEBETT RA. Transplantation of tumors. *In* Methods of Cancer Research (Busch H, ed). New York, Academic Press, vol 1, 1967, pp 143–242.
15. HAAGENSON CD. Diseases of the Breast. W. B. Saunders Co., Philadelphia, 1956.
16. BUCALOSSI P, and VERONESI U. An analysis of some etiological factors in human breast cancer. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 783–789.
17. HESTON E, and VLAHAKIS G. Genetic factors in mammary tumorigenesis. *In* Carcinogenesis: A Broad Critique. Baltimore, Williams and Wilkins Co., 1967, pp 347–363.
18. HARAN-GHERA N. The mechanism of carcinogenesis in breast tumor development in mice. Acta Un Int Cancr 19:765–768, 1963.
19. NANDI S. Host-virus interactions in the mouse mammary tumor system. *In* Carcinogenesis: A Broad Critique. Baltimore, Williams and Wilkins Co., 1967, pp 296–314.
20. WOGLAM WH. The regression of spontaneous mammary carcinoma in the mouse. J Cancer Res 7:379,394, 1922.
21. SMITHERS DW. Some observations on long survival in patients with advanced cancer of the breast. *In* Second International Symposium on Mammary Cancer (Severi L, ed). Italy, Division of Cancer Research, University of Perugia, 1958, pp 311–320.
22. EVERSON TC, and COLE WH. Spontaneous Regression of Cancer. Philadelphia, W. B. Saunders Co., 1966.
23. DMOCHOWSKI L, PRIORI ES, WILLIAMS WC, ET AL. Comparative studies on breast cancer in animals and man. *In* Fundamental Research on Mammary Tumours. J. Mouriquand, Centre d'Etudes Nucléaires et Faculté de Médecine de Grenoble, 1972, pp 351–359.
24. MOORE DH, CHARNEY J, KRAMARSKY B, ET AL. Search for a human breast cancer virus. Nature 229:611–615, 1971.
25. CHARNEY J, and MOORE DH. Neutralization of murine mammary tumor virus by sera of women with breast cancer. Nature 229:627–628, 1971.
26. SANDBERG J, and GOLDIN A. Use of first generation

transplants of a slow growing solid tumor for the evaluation of new cancer chemotherapeutic agents. Cancer Chemother Rep 55:233–238, 1971.

27. Di Marco A, Lenaz L, Casazza AM, et al. Activity of adriamycin (NSC-123127) and daunomycin (NSC–82151) against mouse mammary carcinoma. Cancer Chemother Rep 56:153–161, 1972.
28. Martin DS, Fugmann RA, and Hayworth PE. Surgery, cancer chemotherapy, host defenses and tumor size. J Natl Cancer Inst 29:817, 1962.
29. Martin DS, Hayworth PE, Fugmann RA, et al. Combination therapy with cyclophosphamide and zymosan on a spontaneous mammary cancer in mice. Cancer Res 24:652, 1964.
30 Stolfi RL, Martin DS, and Fugmann RA. Spontaneous murine mammary adenocarcinoma: model system for evaluation of combined methods of therapy. Cancer Chemother Rep 55:239–251, 1971.
31. Hellström I, Hellström KE, Sjögren HW, et al. Demonstration of cell-mediated immunity to human neoplasms of various histological types. Int J Cancer 7:1–16, 1971.

Potentially Useful Combinations of Chemotherapy Detected in Mouse Tumor Systems [1]

Ira Kline [2]

Clinical results indicate that multiple-drug chemotherapy provides an advantage over single-drug treatment (1,2). The murine L1210 leukemia model has been shown to be useful in selecting antitumor agents for clinical use and may be useful in selecting therapeutic synergistic combinations (3).

The studies presented in this report show the results of combination treatment with agents of clinical interest in the L1210 leukemia and Lewis lung experimental models. Also presented are results of studies in which chemical agents were employed to protect the host from the toxic effects of antitumor drugs. Other data indicate the influence of treatment schedules and the effects of various ratios of drug dose levels on the overall efficacy of combination chemotherapy.

Summary of Two-Drug Combinations in Mice With Early or Advanced L1210 Leukemia

The first series of tables summarize the response of mice with L1210 leukemia to various combinations of drugs when treatment was started on Days 1, 3, or 5. Combination chemotherapy was considered superior to single-drug therapy if the percent increase in life-span (%ILS) of the combination was greater than or equal to 50% more than the %ILS of the more effective individual drug.

ICRF-159 [3] was studied in combination with each of 16 drugs (table 1). Enhanced effectiveness was observed with six drugs: adriamycin, daunorubicin, camptothecin, a quinolinium derivative (NSC-113089), *cis*-diamminedichloroplatinum, and cytosine arabinoside (table 1).

Adriamycin or daunorubicin given in combination with ICRF-159 produced ILSs of 172% and 170% respectively in mice with early L1210 leukemia. Additional details of these useful combinations are presented in Dr. R. J. Woodman's report in this issue (4).

In two experiments (table 1) the combination of ICRF-159 plus camptothecin elicited at least a 67% ILS over single-drug treatment. Against advanced subcutaneously (sc) implanted L1210 leukemia, however, the ILS produced by the combination was only 30% greater than that of the maximally effective single-drug therapy.

Against advanced L1210 leukemia, treatment with NSC-113089, *cis*-diamminedichloroplatinum, or cytosine arabinoside given in combination with ICRF-159 elicited increases that were 50% or greater than those produced with maximally effective single-drug therapy (table 1).

Of the remaining ten agents tested in combination with ICRF-159 against early or advanced L1210 leukemia, harringtonine provided a 40% increase over the best single-drug therapy; coralyne, 5-HP, and methotrexate provided a 25%–33% increase; and anguidin, chromomycin A_3, ellipticine, 5-azacytidine, and cyclophosphamide provided a zero to 10% increase (table 1).

Table 1 summarizes the antileukemic effectiveness of NSC-45388 given in combination with each of five agents. In two early L1210 leukemia experiments, combination treatment with adriamycin plus NSC-45388 produced ILSs that were 83% and 89% greater than those produced with maximally effective single-drug therapy. Against advanced L1210 leukemia (Day 5), the combination of NSC-45388 plus adriamycin retained its effectiveness. NSC-45388 given in combination with an imidazole carboxamide analog (NSC-51143) produced an ILS 61%

[1] Supported by contract NO-1-CM-33728 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Microbiological Associates, Inc., Bethesda, Md.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

TABLE 1.—Effectiveness of drug combinations in mice with early or advanced L1210 leukemia

Tumor site*	Drug A	Drug B	Treatment schedule† (days)		% ILS			Effectiveness of combination therapy‡
			Drug A	Drug B	Drug A	Drug B	Drug A + drug B	
ip	ICRF–159	Adriamycin	q3h/1	q3h/1	55	60	172(3)§	+
ip		Daunorubicin	1,5,9	1,5,9	60	20	170(3)	+
ip		Camptothecin	1,5,9	1,5,9	155	112	>565(5)	+
ip		Camptothecin	1,5,9	1,5,9	94	117	184(1)	+
ip		Quinolinium derivative (NSC-113089)	3,7,11	3–11	80	125	180	+
sc		*Cis*-diamminedichloroplatinum	5,9,13	q3h/5,9,13	61	67	161	+
sc		Cytosine arabinoside	5,9,13	5,7,9,11,13	68	100	158	+
ip		Harringtonine	1,5,9	1–9	70	40	110	–
ip		Coralyne	1,5,9	1–9	70	25	95	–
ip		Anguidin	1,5,9	1–9	70	20	80	–
ip		Chromomycin A_3	1,5,9	1,5,9	70	20	75	–
ip		Ellipticine	1	1	75	40	60	–
sc		5-Azacytidine	1,5,9	1,5,9	139	117	139	–
sc		5-HP	5,9,13	5,9,13	78	100	133	–
sc		Camptothecin	5,9,13	5,9,13	60	55	90	–
sc		Methotrexate	5,9,13	5–13	44	83	111	–
sc		Cyclophosphamide	5,9,13	5,12,19	68	279	247	–
ip	NSC-45388	Adriamycin	2–12¶	q3h/1	44	55	144	+
ip		Adriamycin	2–12¶	q3h/1,13	44	61	144	+
ip		Adriamycin	5–17	5	33	33	111	+
ip		Imidazole derivative (NSC-51143)	1–9	1,5,9	67	94	155	+
ip		Camptothecin	3–17	3,10,17	44	100	155	+
ip		Camptothecin	3–11	3,7,11	44	144	167	–
ip		Emetine	1–9	1,5,9	68	63	105	–
ip		5-Azacytidine	1–9	1,5,9	67	167	178	–
sc	Camptothecin	Cyclophosphamide	5,12,19	5,12,19	22	122	244	+
sc		Isophosphamide	5,13,21	5,13,21	22	144	250	+
sc		Methotrexate	5,12,19	5–19	44	83	161	+
sc		Methotrexate	5,12,19	5–19	26	79	116	–

Tumor site*	Drug A	Drug B	Treatment schedule† (days)		% ILS			Effectiveness of combination therapy‡
			Drug A	Drug B	Drug A	Drug B	Drug A + drug B	
ip	5-Azacytidine	Emetine	1,5,9	1,5,9	120	45	175	+
ip		Methotrexate	1,5,9	1,5,9	128	111	144	–
ip		Anhydro-ara-C	1–9	1–9	77	82	109	–
sc	Cytosine arabinoside	NSC-82196	q3h/5,9,13,17	5,12,19	100	95	235	+
ip		Emetine	q3h/1,5,9	1,5,9	322	78	144	–

*ip = 10^5 L1210 ascites cells inoculated ip; sc = 10^6 L1210 ascites cells inoculated sc.

†Days of treatment after tumor inoculation; q3h = injections every 3 hrs for 24 hrs on 1 day only or on every 4th day.

‡+ = % increase in median lifespan is ≥ 50% greater than maximally effective single-drug therapy. – = % increase in median lifespan is < 50% greater than maximally effective single-drug therapy.

§( ) = No. of long-term survivors out of 8 mice.

¶Individual drug treatment from Day 1 to Day 11.

greater than that of the optimal single-drug therapy. Treatment with NSC-45388 given every seventh day and camptothecin given from Day 3 increased the lifespan by 55% over the best individual drug response. Combined treatment with NSC-45388 plus emetine or 5-azacytidine produced increases of 37% and 21% respectively over that of the best single-drug response.

The data presented in table 1 show that cyclophosphamide as well as isophosphamide, an analog of cyclophosphamide, provided superior therapies when given in combination with camptothecin. Weekly treatments with camptothecin plus daily treatments with methotrexate achieved a clearly superior therapy in one experiment, but in a second experiment only a 37% ILS over the maximum response with methotrexate alone was observed (table 1). The combination of 5-azacytidine plus emetine produced an ILS of 175%, which was 55% more effective than the maximum response achieved with single-drug treatment (table 1). Weekly treatments with NSC-82196 plus cytosine arabinoside given every 3 hours for 24 hours every fourth day (q3h/4d) were superior to treatment with either drug alone (table 1).

Selected Agents Combined With a Non-Cell-Cycle-Dependent Agent (Cyclophosphamide) and With an S-Phase Cycle-Dependent Agent (Cytosine Arabinoside)

Each of six agents (1-aminocyclopentanecarboxylic acid, procarbazine, methyl-CCNU, dibromodulcitol, 5-HP, and isophosphamide) was studied in combination with either cyclophosphamide or cytosine arabinoside in mice with advanced sc implanted L1210 leukemia. The results of these studies are summarized in table 2. None of these agents provided a superior therapy when given in combination with cyclophosphamide.

The combination of procarbazine given daily plus cytosine arabinoside given q3h/4d provided an enhanced therapy. In combination with 5-HP, however, the most effective therapy (65% ILS) was achieved with cytosine arabinoside given once on every second day plus 5-HP given q3h/4d. On the reverse schedule (cytosine arabinoside given q3h/4d and 5-HP given once every second day), the ILS was only 40% more than the %ILS with the more effective single drug. Isophosphamide given once on Day 5 plus cytosine arabinoside injected q3h/4d on Days 9–17 increased survival rates 41% more than the best single-drug response. None of the remaining agents provided a better therapy in combination with cytosine arabinoside (table 2).

TABLE 2.—Treatment of advanced L1210 leukemia with agents given in combination with an S-phase-dependent agent (cytosine arabinoside) and with a non-S-phase-dependent agent (cyclophosphamide)

NSC No.	Agent	Cytosine arabinoside plus other agents* Treatment schedule (days)	Cyclophosphamide plus other agents* Treatment schedule (days)	Activity of combination† Cytosine arabinoside plus other agents	Activity of combination† Cyclophosphamide plus other agents
77213	Procarbazine	q3h/5,9,13 + qd/5–13	5 only + qd/6–19	++	–
95441	Methyl-CCNU	q3h/8,12,16 + q3h/7	5,12,19 + qd/5–19	–	–
107392	5-HP	q3h/5,9,13 + q2d/5–13 q2d/5–13 + q3h/5,9,13	5 + q3h/6,10,14,18	+ ++	–
109724	Isophosphamide	q3h/9,13,17‡ + 5 only	5 + 5	+	–
104800	Dibromodulcitol	q3h/5,9,13 + qd/5–13	5,12,19 + qd/5–19	–	–
1026	1-Aminocyclopentanecarboxylic acid	q3h/5,9,13 + qd/5–13	5 + qd/6–19	–	–

*Treatment schedule for cytosine arabinoside or cyclophosphamide is shown first. q3h = injection every 3 hrs for 24 hrs for 1 day only, or on every 4th day; qd = daily treatment; q2d = treatment on every 2nd day.

†++ = % increase in median lifespan of the combination is $\geq 50\%$ greater than maximally effective single-drug therapy; + = % increase in median lifespan of the combination is 25%–49% greater than maximally effective single-drug therapy; – = % increase in median lifespan of the combination is $< 25\%$ greater than maximally effective single-drug therapy.

‡Treatment schedule for cytosine arabinoside given alone, q3h/5,9,13.

Studies to Increase the Selective Toxic Effects of Active Agents

The data summarized in figure 1 indicate that 6-azauridine reversed the antileukemic effect and reduced the toxic effects of 5-azacytidine to the host. Although treatment with 6-azauridine permitted the use of higher dose levels of 5-azacytidine, the therapeutic effect was not superior to treatment with 5-azacytidine alone.

The data presented in figure 2 show that acetylcysteine, an agent without antitumor activity, protected against the toxic effects of isophosphamide, and that the combination produced an enhanced therapeutic effect greater than that seen with isophosphamide given alone to mice with early L1210 leukemia. Isophosphamide given alone to leukemic or nontumored mice on Day 1 only was lethal at a dose level of 500 mg/kg. The optimal dose level for isophosphamide (300 mg/kg) administered alone extended the median survival time (MST) to 23.5 days. When isophosphamide was preceded by acetylcysteine, a dose of 500 mg/kg of isophosphamide was tolerated by the nontumored and leukemic mice and an improved antitumor effect was observed (> 60 days).

Figure 3 illustrates the influence of the metabolite, deoxycytidine, on the toxic effects and antileukemic activity of anhydro-ara-C. Twice-daily treatment with deoxycytidine (75 mg/kg) plus daily treatment with anhydro-ara-C at a dose level of 400 mg/kg produced five of six survivors in nontumored mice. Only one of six nontumored mice survived the 400-mg/kg dose of anhydro-ara-C when given alone. Combined treatment at these dose levels in leukemic mice extended the survival time to 22.5 days as compared with 21 days at the optimal dose level of 200 mg/kg of anhydro-ara-C alone.

Factors Influencing the Efficacy of Combination Chemotherapy

The use of a model mouse tumor system for the evaluation of drug combinations requires

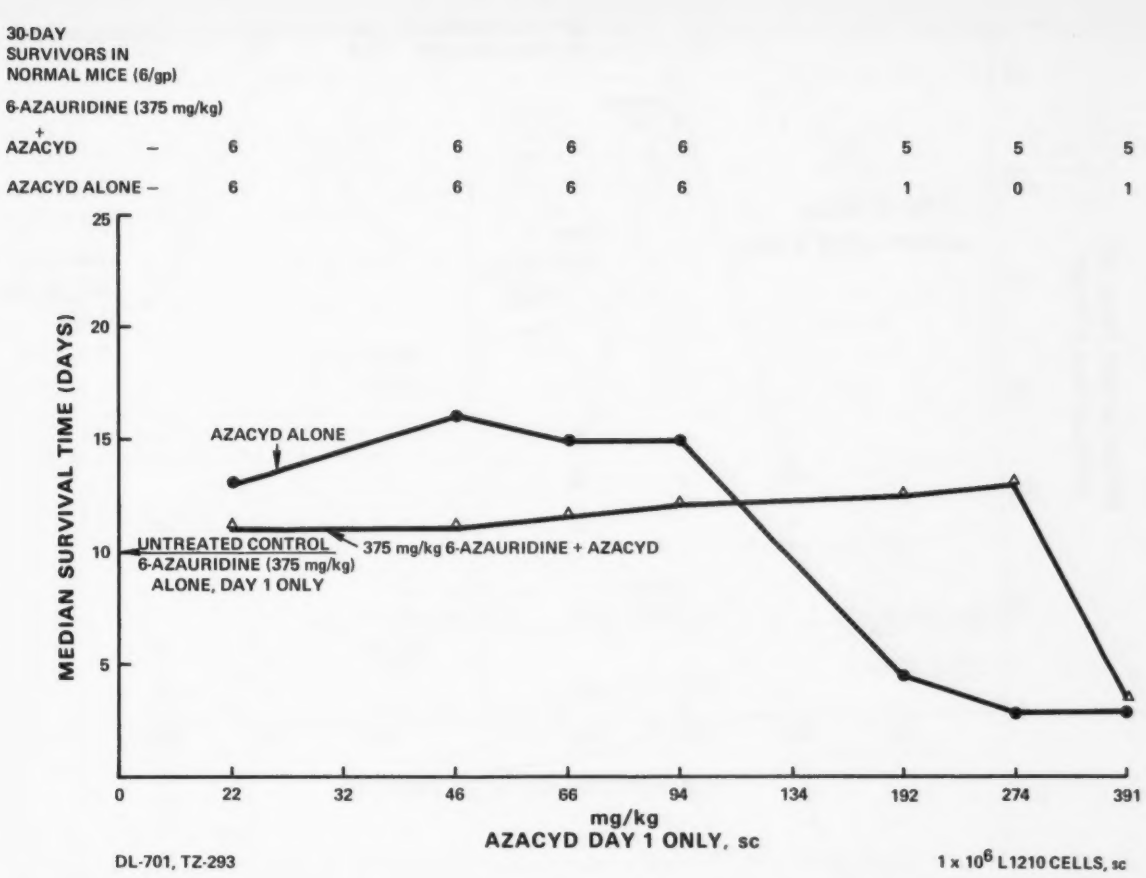


FIGURE 1.—Ability of 6-azauridine to reverse the antileukemic effect and to reduce the toxicity to the host of 5-azacytidine (AZACYD). Eight L1210 leukemic and six nontumored BDF_1 male mice (18–27 g) per treated group and 16 untreated leukemic controls were used. Both drugs were administered sc on Day 1 only.

certain modifications in experimental design (5), eg, there are many schedules of treatment to select from and there are also numerous possible dose-ratios to be considered. There is an optimal treatment level for a combination of drugs just as there is an optimal dose level for a single drug. With this in mind, we employ several dose ratios at logarithmically spaced levels in our combination studies. Figure 4 illustrates the optimal dose-ratio for the synergistic action of methotrexate and 5-fluorouracil (5-FU) against advanced L1210 leukemia (6). In this study, the improved therapy was achieved with an optimal dose level of methotrexate plus a relatively low, nontoxic, and not detectably effective dose of 5-FU at a ratio of 16:1 (5-FU to methotrexate). On the other hand, the data presented in figure 5 show that with 5-FU plus NSC-45388 (7) the most effective therapy was produced by combining 5-FU at its optimal dose level (23 mg/kg) with NSC-45388 at a dose level (39 mg/kg) that was lower than the optimal dose for NSC-45388 alone. The dose-response curve in the right panel of figure 5 shows that the combination of an optimal dose of NSC-45388 with a suboptimal dose of 5-FU was inferior to the combination of an optimal dose of 5-FU and a suboptimal dose of NSC-45388.

The treatment schedule used also influences the effectiveness of combination chemotherapy. The optimal schedule for a drug given alone is not necessarily the best schedule for combination chemotherapy. In the evaluation of

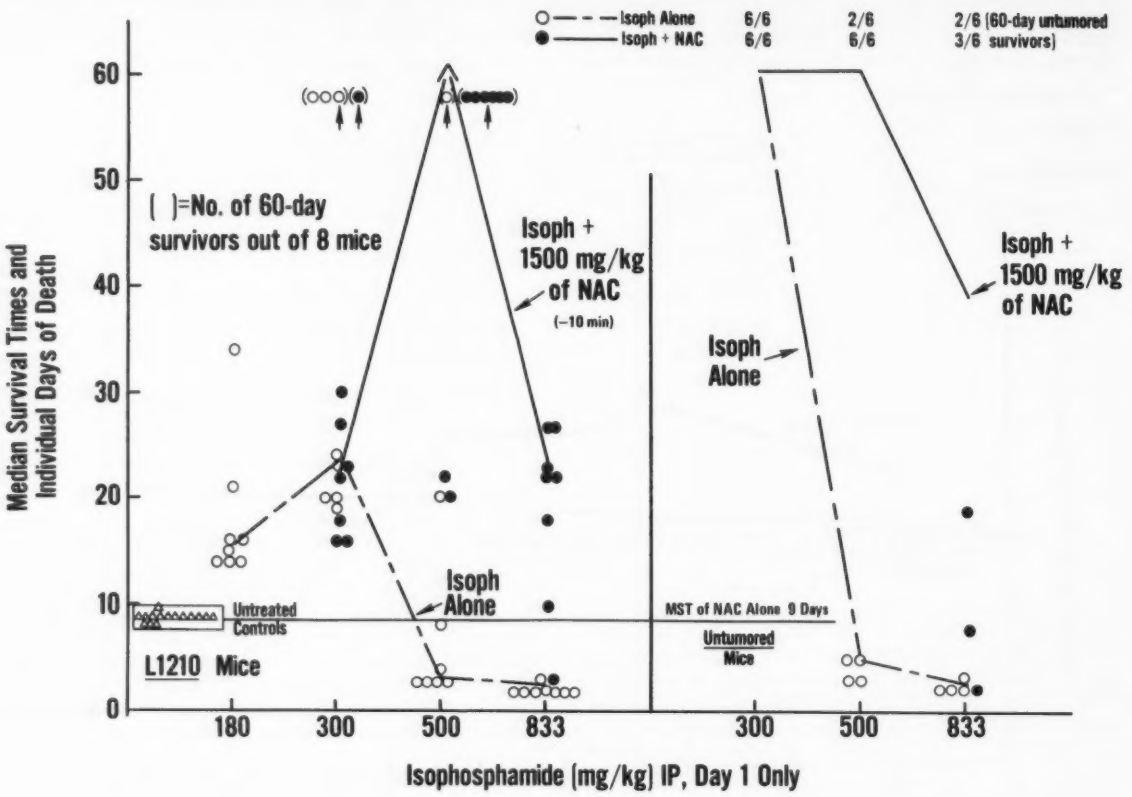


FIGURE 2.—Ability of acetylcysteine (NAC) to reduce the toxicity and enhance the L1210 efficacy of isophosphamide (Isoph). Acetylcysteine was given 10 minutes before isophosphamide. Eight leukemic and six nontumored BDF, male mice (18–25 g) per treated group and 16 untreated leukemic controls were used.

combination chemotherapy, the single drugs must be employed on optimal schedules of treatment, and these schedules, in addition to others, are used in the drug combinations. The data shown in figure 6 illustrate the influence of variations in treatment schedules on the therapeutic effectiveness of the combination of cyclophosphamide and camptothecin. In mice with advanced L1210 leukemia, weekly treatment with cyclophosphamide plus weekly treatment with camptothecin produced a therapeutic effect 122% greater than that of cyclophosphamide given alone. In contrast, the combination of cyclophosphamide given on Day 5 only plus camptothecin injected on Days 5, 9, and 13 (the more effective schedule for this drug alone) did not appreciably improve upon single-drug therapy.

Combination Chemotherapy Against Established Lewis Lung Carcinoma

In addition to combination drug studies against the rapidly proliferating L1210 system, such therapeutic studies are also carried out in the slow-growing metastasizing Lewis lung model. Table 3 presents the data from a combination study which employed cyclophosphamide, an agent that is effective in inhibiting the primary tumor and metastases, plus ICRF-159, a drug that inhibits metastatic growth with a limited effect on the primary tumor. Combined treatment with cyclophosphamide given on Day 5 only plus ICRF-159 injected on Days 5, 9, and 13 produced an ILS of 163% which was 76% greater than the effect elicited by cyclophosphamide alone. The data summarized in table 4

TABLE 3.—Combination treatment of established intramuscularly implanted Lewis lung carcinoma with cyclophosphamide plus ICRF-159*

Agent	Treatment schedule (ip)	Dose range (mg/kg/ injection)	Optimal dose (mg/kg/ injection)	MST	Range of deaths (days)	%ILS†	No. of 60-day survivors/ total No. treated
Cyclophosphamide	Day 5 only	108–500	180	38.5	28–>70	87	1/8
ICRF-159	Days 5, 9, 13	50–400	200	22.5	13–34	9	0/8
Cyclophosphamide	Day 5 only	108–300	180				
+	+	+	+	54	22–>70	163	3/8
ICRF-159	Days 5, 9, 13	12.5–400	100				
Untreated controls				20.5	13–31		0/16

*C57BL/6 male mice (18–24 g) inoculated im with 2×10^6 cells.
†Percent increase in MST over untreated controls.

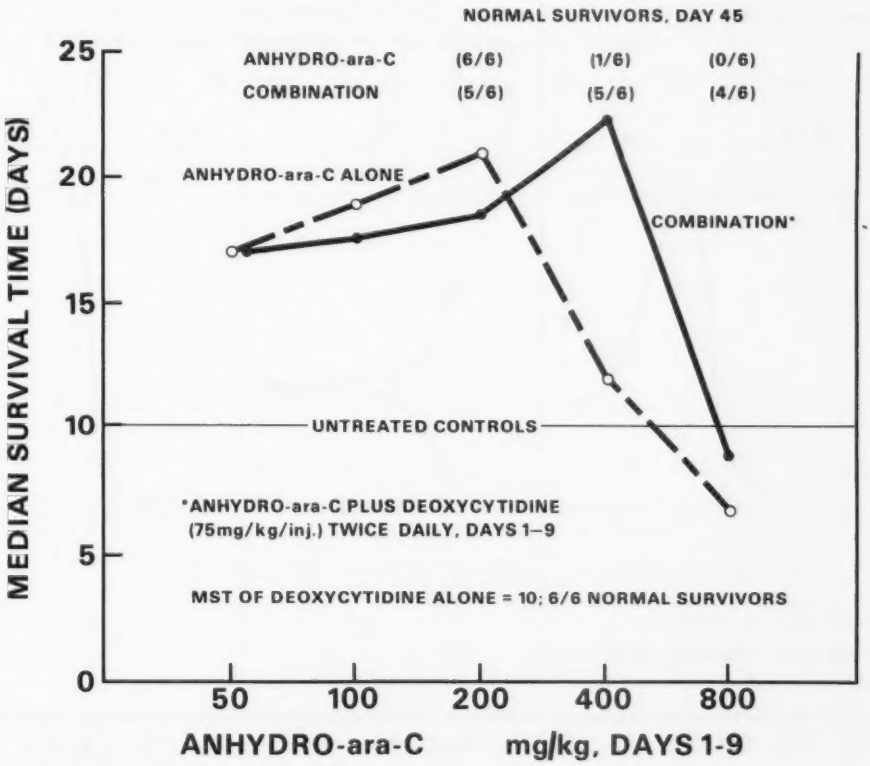


FIGURE 3.—Ability of deoxycytidine to protect the host from the toxicity of anhydro-ara-C. Eight leukemic and six nontumored BDF, male mice (19–26 g) per treated group and 16 untreated leukemic controls were used. The leukemic mice were inoculated sc with 1×10^6 cells and sc treatment was started on Day 1 after tumor implantation.

TABLE 4.—Combination therapy of established intramuscularly implanted Lewis lung carcinoma with procarbazine plus bleomycin*

Agent	Dose range (mg/kg/ injection)	Optimal dose (mg/kg/ injection)	MST	Range of deaths (days)	%ILS	No. of survivors/ total No. treated
Procarbazine	39–300	65	22.5	7–41	2	0/8
Bleomycin	3.0–39	8.4	32	6–46	45	0/8
Procarbazine + Bleomycin	23–180 + 1.8–23	65 + 14	38.5	21–49	75	0/8
Untreated controls			22	17–31		0/16

*BDF_1 male mice (21–26 g) inoculated im with 2×10^6 cells; ip treatment, daily, Days 5–13.

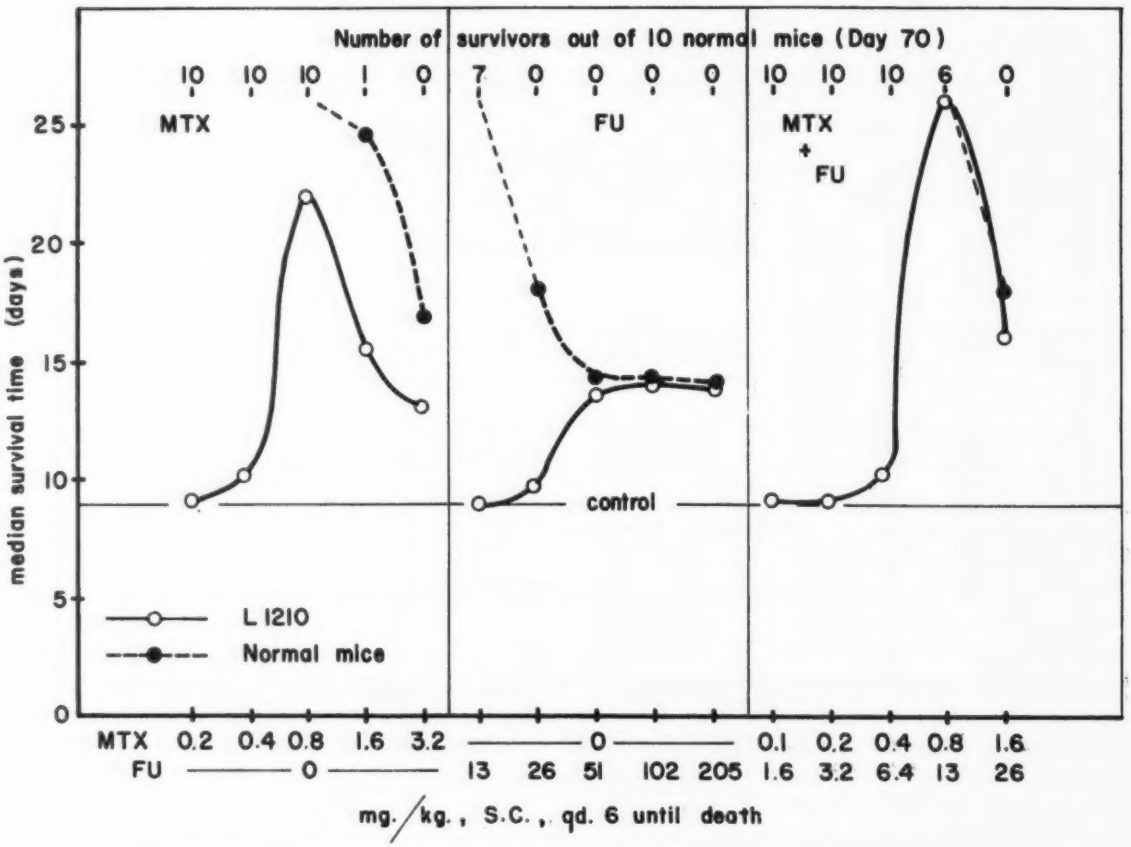


FIGURE 4.—Dose response of normal and L1210 leukemic mice to treatment with 5-FU and methotrexate (MTX), alone and in combination.

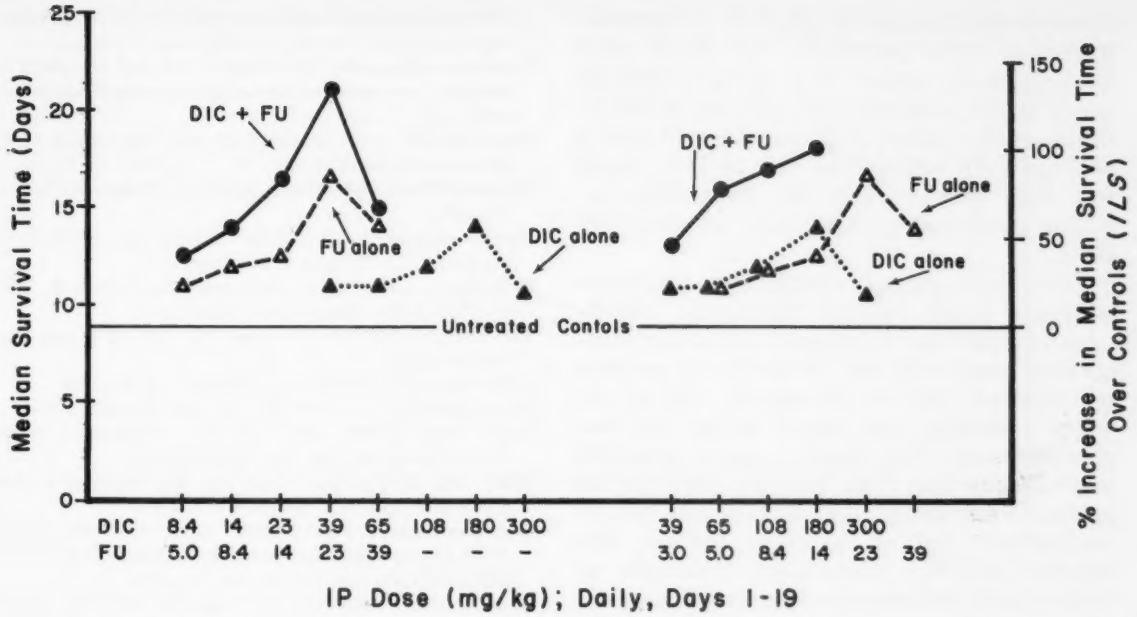

Median Survival Time (Days)
% Increase in Median Survival Time Over Controls (ILS)
DIC + FU
FU alone
DIC alone
Untreated Contols
DIC 8.4 14 23 39 65 108 180 300
FU 5.0 8.4 14 23 39 – – –
39 65 108 180 300
3.0 5.0 8.4 14 23 39
IP Dose (mg/kg); Daily, Days 1-19

FIGURE 5.—Response of L1210 leukemic mice to combination daily treatment with NSC-45388 (DIC) and 5-FU. The inoculum, 1×10^5 L1210 ascites cells, was implanted ip into BDF_1 male mice which weighed 19–26 g at the beginning of the experiment. Eight mice were used in the treated groups and 16 mice in the untreated groups.

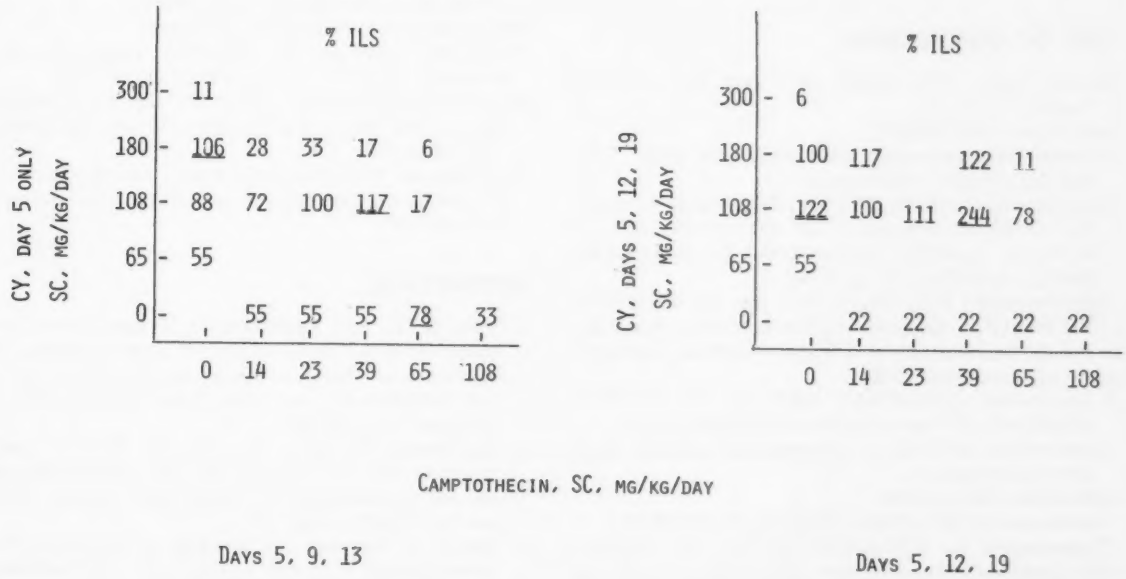


FIGURE 6.—Influence of treatment schedule on the efficacy of combination treatment of advanced sc implanted L1210 leukemia with cyclophosphamide (CY) plus camptothecin. The inoculum, 1×10^6 L1210 ascites cells, was implanted sc into BDF_1 (18–25 g) mice. Eight mice were used in the treated groups and 16 mice in the untreated groups. Sc treatment was initiated on Day 5 after tumor inoculation.

illustrate the therapeutic effect of combination treatment with bleomycin (an agent with marked activity against the Lewis lung primary tumor as well as metastases) plus procarbazine. In this study, daily treatment with both agents on Days 5–13 achieved an ILS of 75% which was 30% greater than the therapeutic response produced by bleomycin administered alone.

This report presents examples of drug combinations which provide therapeutic advantages in experimental leukemia and solid metastasizing tumor systems. In certain synergistic combinations, one of the agents was an inactive compound (eg, acetylcysteine and isophosphamide). Other inactive agents appeared to protect the host from the toxic effects of the antitumor compound (eg, 6-azauridine and 5-azacytidine), but no enhanced therapy was detected with the combination. Examples of factors which influence the effectiveness of combinations of drugs, such as drug dose-ratios and schedules of treatment, were also presented. Hopefully, studies with experimental test systems will lead to the more effective use of multiple chemotherapy in the clinic.

LIST OF COMPOUNDS

Acetylcysteine: NSC-111180; CAS reg. No. 616-91-1; *N*-acetyl-L-cysteine

Adriamycin: NSC-123127

1-Aminocyclopentanecarboxylic acid: NSC-1026; CAS reg. No. 52-52-8; cycloleucine

Anguidin: NSC-141537; CAS reg. No. 22135-66-6; spiro-[2,5-methano-1-benzoxepin-10,2′-oxirane]-5α(6*H*)-methanol, 2,3,4,5,7,9α-hexahydro-3,4-dihydroxy-5,8-dimethyl-, 4,5α-diacetate; spirobus

Anhydro-ara-C: NSC-129220; CAS reg. No. 10212-28-9; 6*H*-furo[2′,3′,:4,5]oxazolo[3,2-a]pyrimidine-2-methanol, 2,3,3a,9a-tetrahydro-3-hydroxy-6-imino-, monoacetate (salt); cyclocytidine

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

6-Azauridine: NSC-32074; *as*-triazine-3,5(2*H*,4*H*)-dione, 2-β-D-ribofuranosyl-

Bleomycin: NSC-125066

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

Chromomycin A_3: NSC-58514; CAS reg. No. 7059-24-7

***Cis*-diamminedichloroplatinum:** NSC-119875; CAS reg. No. 15663-27-1

Coralyne: NSC-96349; CAS reg. No. 6872-73-7; dibenzo-[a,g]quinolizinium, 2,3,10,11-tetramethoxy-8-methyl-, chloride

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

Deoxycytidine: NSC-83251; cytidine, 2′-deoxy-, hydrochloride

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1, 6-dideoxy-

Ellipticine: NSC-71795; CAS reg. No. 519-23-3; 6*H*-pyrido[4,3-b]carbazole, 5,11-dimethyl-

Emetine: NSC-33669; CAS reg. No. 316-42-7; emetine, hydrochloride

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Harringtonine: NSC-124147; CAS reg. No. 25302-09-4

5-HP: NSC-107392; CAS reg. No. 19494-89-4; 5-hydroxypicolinaldehyde thiosemicarbazone

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4′-propylenedi-, (±)-

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-thriazeno)-

NSC-51143: 1*H*-pyrazole[2,3-a]imidazole

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-

NSC-113089: quinolinium, 6-amino-1-ethyl-4-[*p*-[[*p*-[(1-ethylpyridinium-4-yl)-amino]phenyl]carbamoyl]anilino]-, dibromide

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

REFERENCES

1. FREI E III, and FREIREICH EJ. Progress and perspectives in the chemotherapy of acute leukemia. *In* Advances in Chemotherapy (Goldin A, Hawking F, and Schnitzer RJ, eds). New York, Academic Press, 1965, vol 2, pp 269–298.
2. HENDERSON ES, and SAMAHA RJ. Evidence that drugs in multiple combinations have materially advanced the treatment of human malignancies. Cancer Res 29:2272–2280, 1969.
3. GOLDIN A, SANDBERG JS, HENDERSON ES, ET AL. The chemotherapy of human and animal acute leukemia. Cancer Chemother Rep 55:309–507, 1971.
4. WOODMAN RJ. Enhancement of antitumor effectiveness of ICRF-159 (NSC-129943) against early L1210 leukemia by combination with *cis*-diamminedichloroplatinum (NSC-119875) or daunomycin (NSC-

82151). Cancer Chemother Rep Part 2, vol 4:45–52, 1974.

5. VENDITTI JM, and GOLDIN A. Drug synergism in antineoplastic chemotherapy. *In* Advances in Chemotherapy (Goldin A, and Hawking F, eds). New York, Academic Press, 1964, vol 1, pp 397–498.
6. KLINE I, VENDITTI JM, MEAD JAR, ET AL. The antileukemic effectiveness of 5-fluorouracil and methotrexate in the combination chemotherapy of advanced leukemia L1210 in mice. Cancer Res 26:848–852, 1966.
7. KLINE I, WOODMAN RJ, GANG M, ET AL. Enhanced response of leukemic (L1210) mice to combination chemotherapy with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) and 5-fluorouracil (NSC-19893). Cancer 27:1363–1368, 1971.

Enhancement of Antitumor Effectiveness of ICRF-159 (NSC-129943) Against Early L1210 Leukemia by Combination With *Cis*-diamminedichloroplatinum (NSC-119875) or Daunomycin (NSC-82151) [1]

R. J. Woodman [2]

In the preceding presentation, the identification of therapeutically synergistic combinations of antitumor drugs from a large number of possible candidates using mouse tumor systems was described. Further characterization of these synergistic combinations is usually desirable in order to provide additional information that might help the clinician in the evaluation and clinical application of the results from animal studies.

The basic approach toward this objective has been to increase the challenge of the tumor to the host by either extending the period between tumor cell inoculation and initiation of treatment or manipulating the sites of tumor implantation and drug injection. Two examples of this type of further investigation of synergistic drug combinations will be described for the L1210 leukemia system.

The first example is the combination of ICRF-159 with *cis*-diamminedichloroplatinum. The structures of these two compounds are shown in figure 1. ICRF-159, a sodium edetate [3] derivative formed by ring closure of the diimide of ethylenediaminetetraacetic acid,[4] is a water-insoluble drug that is active against L1210 leukemia when given either orally or intraperitoneally (ip). NSC-119875, a complex salt of platinum that is devoid of carbon, has an unusual structure for an antitumor agent.

The synergistic antitumor effect of these two substances against early L1210 leukemia was shown in the preceding report. Before this drug combination was evaluated in advanced L1210 leukemia, the optimal schedule for treatment of early L1210 leukemia was established for each agent. Table 1 shows that for *cis*-diamminedichloroplatinum, optimal antitumor effect is relatively independent of treatment schedule, whereas for ICRF-159, the greatest extension of lifespan is obtained when the drug is given every fourth day. Treatment with ICRF-159 once or every 3 hours on Days 1, 5, and 9 after ip inoculation with 10^5 L1210 leukemic cells in combination with *cis*-diamminedichloroplatinum injected once on Days 1, 5, and 9 gave the greatest degree of therapeutic synergism. These treatment schedules were applied to the advanced L1210 tumor system in which 10^6 leukemic cells were injected subcutaneously (sc) and treatment was started 5 days later. Figure 2 shows that the therapeutic effect against advanced systemic disease was significantly enhanced with this combination of drugs: the increase in lifespan obtained with the combination was about twice that obtained with the more effective single agent *cis*-diamminedichloroplatinum. With either schedule of ICRF-159 administration, the optimal combination dose for *cis*-diamminedichloroplatinum was about half the optimal dose for treatment with *cis*-diamminedichloroplatinum alone. This indicates that there is some additive toxicity with the combination treatment.

This question of optimal dose for a drug for single-drug treatment and combination treatment is of interest with respect to the second drug combination to be described, ie, the com-

[1] ICRF-159: CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4'-propylenedi-, (±)-.

Cis-diamminedichloroplatinum: CAS reg. No. 15663-27-1; *cis*-Pt(II).

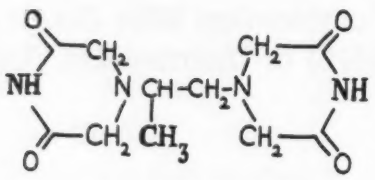
Daunomycin: CAS reg. No. 23541-50-6; daunorubicin (NSC-83142).

[2] Microbiological Associates, Inc., Bethesda, Md.

[3] Sodium edetate: CAS reg. No. 64-02-8; acetic acid, (ethylenedinitrilo)tetra-, tetrasodium salt; Versene.

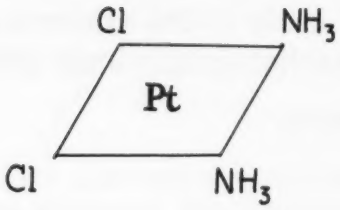
[4] Ethylenediaminetetraacetic acid: NSC-2760; CAS reg. No. 6381-92-6; acetic acid, (ethylenedinitrilo)-tetra-, disodium salt, dihydrate; EDTA; disodium edetate.

NSC 129943



ICRF-159 1,2-di(3,5-dioxo-piperazine-1-yl) propane

NSC 119875



cis platinum (II) diamminodichloride

FIGURE 1.—Structures of ICRF-159 and *cis*-diamminedichloroplatinum.

bination of ICRF-159 with either of the anthracycline antibiotics daunomycin or adriamycin. (Chemical structures are shown in fig 3.) This combination was first suggested by the work of Herman et al (1) who discovered that ICRF-159 or sodium edetate reduced the toxicity of daunomycin or adriamycin to the isolated dog heart. Although our toxicologic studies in normal mice did not include measurements of cardiotoxicity, we found that the

TABLE 1.—Schedule dependency of ICRF-159 and *cis*-diamminedichloroplatinum given ip alone and in combination against early L1210 leukemia*

Experiment No.	Drug	% ILS† after treatment administered— Once, Day 1 only	Once, Days 1, 5, and 9	q3h, Days 1, 5, and 9‡	Daily, Days 1–9
1	*Cis*-diamminedichloroplatinum alone	55	78	111	78
2	ICRF-159 alone	33	133	161	72
	Cis-diamminedichloroplatinum alone	61	78	—	—
3	ICRF-159 alone	50	122	206	—
	ICRF-159 + *cis*-diamminedichloroplatinum	72	300	211	—
4	ICRF-159 alone	—	—	140	—
	ICRF-159 + *cis*-diamminedichloroplatinum	—	—	>560	—

*10^5 L1210 leukemia cells implanted ip.
†% ILS = percent increase in lifespan.
‡Schedule for ICRF-159 only; *cis*-diamminedichloroplatinum was given once on Days 1, 5, and 9.

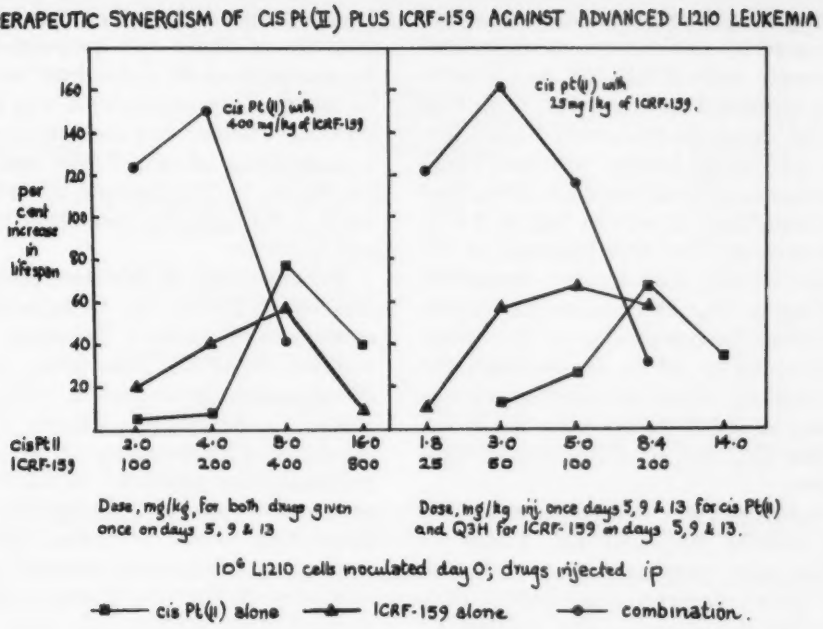

THERAPEUTIC SYNERGISM OF CIS Pt(II) PLUS ICRF-159 AGAINST ADVANCED L1210 LEUKEMIA
cis Pt(II) with 400 mg/kg of ICRF-159
cis pt(II) with 25 mg/kg of ICRF-159.
per cent increase in lifespan
160
140
120
100
80
60
40
20
cisPtII 2·0 4·0 8·0 16·0 1·8 3·0 5·0 8·4 14·0
ICRF-159 100 200 400 800 25 50 100 200
Dose, mg/kg, for both drugs given once on days 5, 9 & 13
Dose, mg/kg inj. once days 5, 9 & 13 for cis Pt(II) and Q3H for ICRF-159 on days 5, 9 & 13.
10⁶ L1210 cells inoculated day 0; drugs injected ip
cis Pt(II) alone
ICRF-159 alone
combination.

FIGURE 2.—Therapeutic enhancement by combination chemotherapy with *cis*-diammine-dichloroplatinum (*cis*-Pt[II]) and ICRF-159 against advanced L1210 leukemia.

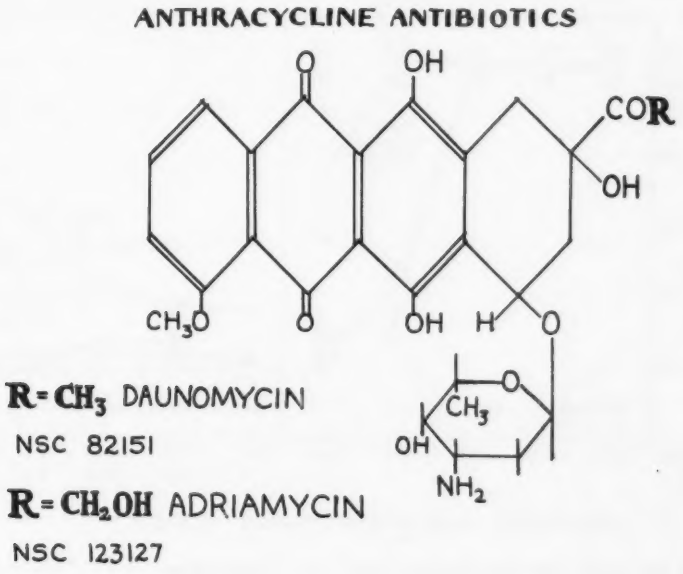


FIGURE 3.—Structures of daunomycin and adriamycin.

maximum dose of ip injected daunomycin that could be tolerated by nonleukemic mice treated ip simultaneously with ICRF-159 was greatly increased. In addition, ip treatment with this combination of drugs in mice with L1210 leukemia gave distinctly longer survival times than did treatment with either drug alone (*see* fig 4). It is important to note in figure 4 that the greatest survival time was obtained in the leukemic mice in the combination treatment group receiving a dose of daunomycin higher than the optimal therapeutic dose for treatment with daunomycin alone. In contrast, the optimal therapeutic dose of *cis*-diamminedichloroplatinum in combination with ICRF-159 was lower than that for *cis*-diamminedichloroplatinum alone.

Further investigation of the reversal of daunomycin toxicity by ICRF-159 (table 2) indicates that the reversal occurs whether ICRF-159 is given several hours before daunomycin or after daunomycin. The reversal of toxicity was little influenced by incubating a mixture of ICRF-159 suspended in dissolved daunomycin at 37 C for half an hour prior to injection. This experiment was included to determine whether daunomycin could react with a suspension of ICRF-159 under physiologic conditions in the absence of enzyme action to form a daunomycin derivative that was virtually nontoxic.

Enhancement of the therapeutic effect of ip injected ICRF-159 by ip injected daunomycin against early L1210 leukemia was observed whether ICRF-159 was given 4 hours before daunomycin or 6 hours after daunomycin (table 3). In one experiment, ICRF-159 was incubated with daunomycin prior to injection. Preincubation appeared to diminish the therapeutic effect of the combination. This effect is interesting when compared with the effect of preincubation of daunomycin and ICRF-159 mixtures on toxicity to nonleukemic mice. It suggests that daunomycin may undergo some

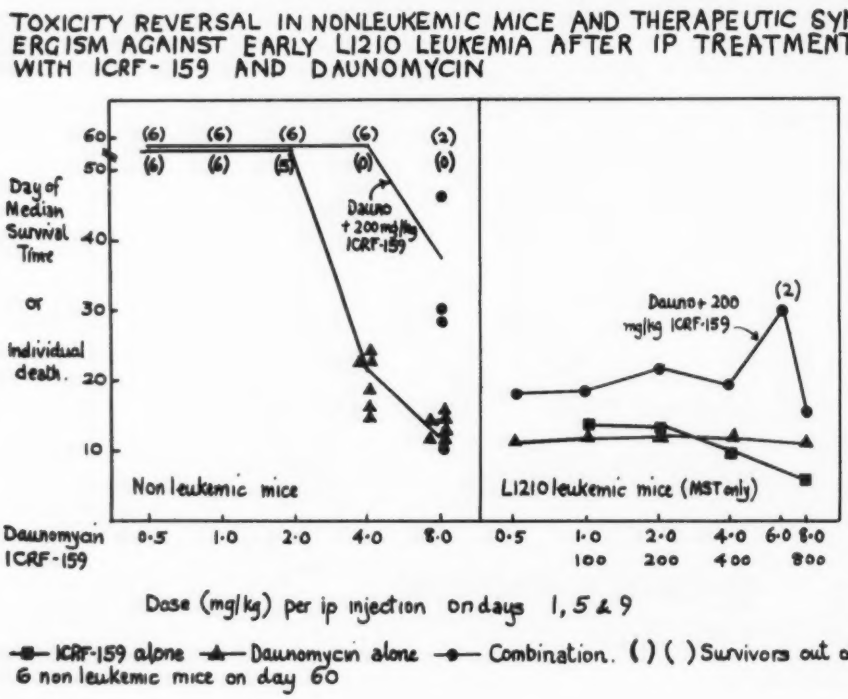

TOXICITY REVERSAL IN NONLEUKEMIC MICE AND THERAPEUTIC SYNERGISM AGAINST EARLY L1210 LEUKEMIA AFTER IP TREATMENT WITH ICRF-159 AND DAUNOMYCIN
Day of Median Survival Time or Individual death.
Nonleukemic mice
L1210 leukemic mice (MST only)
Dauno + 200 mg/kg ICRF-159
Daunomycin
ICRF-159
Dose (mg/kg) per ip injection on days 1, 5 & 9
ICRF-159 alone
Daunomycin alone
Combination. ( ) ( ) Survivors out of 6 nonleukemic mice on day 60

FIGURE 4.—Toxicity reversal and therapeutic enhancement against early L1210 leukemia by combination treatment with ICRF-159 and daunomycin.

TABLE 2.—Toxicity of daunomycin in nonleukemic mice when injected alone or with ICRF-159 under various conditions

Experiment No.	Conditions of drug injection	Maximum tolerated dose of daunomycin (mg/kg) per ip injection, Days 1, 5, and 9	
		Daunomycin alone	Daunomycin with ICRF-159*
1	ICRF-159 injection given 4 hrs before daunomycin	2.5	5.0
2	ICRF-159 injection given 5 min before daunomycin	1.8	5.0
	ICRF-159 injection given 6 hrs after daunomycin	1.8	3.0
3	Drugs injected without preincubation	1.8	5.0
	Drugs preincubated before injection†	3.0	8.3

*Maximum tolerated dose of daunomycin with optimal ICRF-159 dose for toxicity reversal.

†Drugs were incubated (separately or mixed) for 30 minutes at 37 C in Klucel vehicle prior to injection. ICRF-159 was in suspension maintained by shaking.

TABLE 3.—Therapeutic effectiveness of ICRF-159 and daunomycin given ip alone and in combination against early ip implanted L1210 leukemia under various conditions of injection *

Experiment No.	Conditions of drug injection	% ILS† after treatment with—		
		ICRF-159 alone	Daunomycin alone	ICRF-159 plus daunomycin‡
1	ICRF-159 and daunomycin given simultaneously	155	22	>655 (4)
2	ICRF-159 and daunomycin given simultaneously	44	44	233
3	ICRF-159 given 4 hrs before daunomycin	94	33	>277 (4)
4	ICRF-159 and daunomycin given simultaneously	60	20	140
	ICRF-159 given 6 hrs after daunomycin	60	20	170
5	Drugs injected without preincubation	78	28	261
	Drugs preincubated 30 min at 37 C before injection	106	33	128

*Both drugs injected once on Days 1, 5, and 9.

†Percent increase in lifespan at optimal dose.

‡Numbers in parentheses indicate number of animals surviving on Day 60 (there were eight mice in each treatment group).

reaction with ICRF-159 in vitro that inhibits the synergistic antitumor activity of these two compounds.

Although the chemical structure of daunomycin is very similar to that of adriamycin (fig 3), the maximum tolerated dose of ip injected adriamycin could not be increased by concomitant treatment with ICRF-159. How-

ever, therapeutic synergism did occur with adriamycin and ICRF-159 against early ascitic L1210 leukemia (table 4).

At this stage of our study, Lenaz and Di Marco reported (2) that the primary cause of death in mice treated ip with toxic doses of either daunomycin or adriamycin was peritonitis. Mice could tolerate higher doses of either agent if treatment was administered intravenously (iv) or sc. Therefore, further experiments were conducted to study the effect of the injection routes of both L1210 leukemia cells and drug combinations on reversal of toxicity and enhancement of therapeutic effect. The results in table 5 show that nonleukemic mice will tolerate ten times more daunomycin when it is injected sc than when it is injected ip. Furthermore, although the maximum tolerated sc dose of daunomycin could not be increased by concomitant treatment with ICRF-159, the therapeutic effect of ip injected ICRF-159 against early ascitic L1210 leukemia was still enhanced by sc injected daunomycin. The data in table 6 show the effect of varying the site of L1210 tumor inoculation and the route of drug injection on the therapeutic synergism of ICRF-159 plus daunomycin or adriamycin. Therapeutic synergism was observed when the tumor cells were inoculated ip or iv, but it was not observed when they were inoculated sc. Therapeutic synergism was also observed when mice inoculated intracranially with L1210 leukemia cells were treated with ICRF-159 plus daunomycin (ip) on Days 3, 7, and 11. The results in table 6 also show that for ip implanted tumors, therapeutic synergism is observed regardless of the route of drug administration.

TABLE 4.—Therapeutic synergism of ICRF-159 plus adriamycin injected ip on various treatment schedules against early (ip) L1210 leukemia

	% ILS* after treatment with—		
Injection schedule	ICRF-159 alone	Adriamycin alone	ICRF-159 plus adriamycin†
Both drugs injected once, Day 1	33	39	88
Both drugs injected once, Days 1, 5, and 9	61	139	228 (1)
Both drugs injected q3h, Day 1 only	61	55	172 (3)
	60	60	>560 (6)
Adriamycin injected q3h, Day 1 only and	55	30	105
ICRF-159 injected once, Days 1, 5, and 9	60	60	>567 (5)

*Increases in lifespan obtained at optimal dose.

†Numbers in parentheses indicate number of animals surviving on Day 60 (there were eight mice in each group).

TABLE 5.—Effect of route of administration of daunomycin given with ICRF-159 on toxicity and therapeutic effectiveness against early (ip) L1210 leukemia

	Maximum tolerated dose*		% ILS after treatment with—		
Route of daunomycin injection	Daunomycin alone	Daunomycin in combination	Daunomycin alone	ICRF-159 alone†	Daunomycin plus ICRF-159
Sc	12.5	12.5	28	50	144
Ip	1.25	10.0	44	50	150

*Dose (mg/kg) of daunomycin injected by route shown on Days 1, 5, and 9 in nonleukemic mice.

†ICRF-159 injected ip on Days 1, 5, and 9.

TABLE 6.—Effect of varying tumor site and route of drug administration on the survival of mice treated with ICRF-159 plus an anthracycline (daunomycin or adriamycin)

Experiment No.	Site of tumor implantation*	Route of drug administration†		Anthracycline used	% ILS after treatment with—		
		ICRF-159	Anthracycline		ICRF-159	Anthracycline	ICRF-159 and anthracycline
1	Ip	Ip	Ip	Daunomycin	60	20	170
	Ip	Ip	Ip	Adriamycin	139	61	228
2	Ip	Ip	Ip	Daunomycin	50	44	150
	Ip	Ip	Sc	Daunomycin	50	28	144
3	Ip	Ip	Ip	Daunomycin	78	31	144
	Ip	Oral‡	Ip	Daunomycin	84	31	126
4	Sc	Ip	Ip	Daunomycin	61	0	78
	Sc	Ip	Sc	Daunomycin	61	17	67
5	Sc	Oral‡	Sc	Daunomycin	88	39	111
6	Sc	Oral§	Iv	Adriamycin	150	39	128
7	Iv	Ip	Iv	Adriamycin	111	61	190
8	Ic	Ip	Ip	Daunomycin	55	6	83

*Ip inoculum = 10^5 L1210 leukemia cells; sc inoculum = 10^6 cells; iv inoculum = 10^4 cells; ic (intracranial) inoculum = 5×10^5 cells.

†All treatments were given on Days 1, 5, and 9 except in experiment 8 where treatment was given on Days 3, 7, and 11.

‡Animals fasted.

§Animals not fasted.

Daunomycin did not significantly enhance the effectiveness of ICRF-159 against ascitic L1210 leukemia when the start of treatment was delayed to Day 3 (table 7), but with the combination of adriamycin plus ICRF-159 (both injected ip every fourth day beginning on Day 5), there was some evidence of therapeutic synergism in the same tumor system. It is interesting to note that although there was some therapeutic synergism with daunomycin plus ICRF-159 given from Day 3 against the intracranially inoculated tumor (5×10^5 L1210 cells), there was no therapeutic synergism when the combination was given on the same treatment schedule against ip inoculated cells.

With the first drug combination described, ie, ICRF-159 plus *cis*-diamminedichloroplatinum, therapeutic synergism was still manifested at the advanced stage of disease regardless of the site of tumor inoculation or the route of drug injection. With the second

TABLE 7.—Therapeutic effect of ICRF-159 plus daunomycin or adriamycin against advanced ip implanted L1210 leukemia*

Anthracycline used	Ip treatment schedule	% ILS after treatment with—		
		ICRF-159 alone	Anthracycline alone	ICRF plus anthracycline
Adriamycin	Days 5, 9, and 13	55	44	72
Daunomycin	Days 3, 7, and 11	144	33	150

*10^5 L1210 cells implanted ip on Day 0.

combination, therapeutic synergism could be prevented even in the early stages of disease by alteration of the site of tumor inoculation.

Early ip treatment of mice inoculated ip with L1210 leukemia is effective in identifying potentially useful drug combinations; however, as shown by our studies, factors such as site of tumor inoculation, treatment route, and stage of disease should be investigated during further evaluation of such drug combinations.

REFERENCES

1. HERMAN EH, MHATRE RM, LEE IP, ET AL. Prevention of the cardiotoxic effects of adriamycin and daunomycin in the isolated dog heart. Proc Soc Exp Biol Med 140:234–239, 1972.
2. LENAZ L, and DI MARCO A. On the reported lack of effectiveness of daunomycin (NSC-82151) and adriamycin (NSC-123127) on solid tumors. Cancer Chemother Rep 56:431–432, 1972.

Combination Chemotherapy for Spontaneous AKR Lymphoma [1]

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SUMMARY

Since several advanced, clinically recognizable, transplantable murine leukemias are curable with currently available anticancer drugs, chemotherapy trials against spontaneous leukemia-lymphoma in laboratory animals are indicated. Accordingly, we and others are using the spontaneous lymphoma of AKR mice as a laboratory model. This disease appears in a high proportion of AKR mice between 6 and 12 months of age and can be accurately diagnosed clinically after it becomes quite advanced. Median lifespan from diagnosis to death is less than 3 weeks (17 days in our laboratory), and the disease has been shown to respond to a number of drugs useful in treating leukemia-lymphoma in man. Clinical remission can be consistently induced with vincristine plus prednisone in 50% or more of mice with spontaneous lymphoma, and even higher clinical response rates occur with cyclophosphamide plus either an active nitrosourea or arabinosylcytosine (ara-C). An approximate 5-$\log_{10}$ reduction in viable lymphoma cells in the thymus glands can be obtained by any of these three remission-induction drug combinations. Treatment of AKR mice bearing spontaneous clinically diagnosed lymphoma with ara-C plus cyclophosphamide or cyclophosphamide plus an active nitrosourea increases lifespan significantly and consistently, probably by reducing the lymphoma stem cells to very small numbers. This is indicated by the fact that over 80% of young AKR mice that are implanted intravenously with one to ten viable lymphoma cells and subsequently die of the transplanted disease die within 60 days of tumor cell implant. The best treatment regimens with ara-C plus cyclophosphamide or cyclophosphamide plus methyl-CCNU yield significant numbers of 60-day posttherapy survivors. Very good therapeutic responses, based on increased lifespan, bioassay for viable lymphoma cells in selected organs and tissues, and survivors 60 days after the last treatment, were observed with remission induction using vincristine plus prednisone followed by sequential cyclophosphamide and ara-C (in either order) or ara-C followed by an active nitrosourea. Limited trials using methotrexate, adriamycin,

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[2] Southern Research Institute, Birmingham, Ala.

[3] We wish to acknowledge the very competent help of Jean I. McKinstry in establishing clinical diagnosis and conducting chemotherapy lifespan trials; Maxie H. Witt and Rosalie P. Holcombe in conducting the bioassays for tumor cells; and Anne D. Brazier and Nadine D. Hood for the extensive data storage, retrieval, and analysis required in these studies.

palmO-ara-C, or palmO-ara-C plus 6-thioguanine as maintenance therapy following remission induction with vincristine plus prednisone failed to demonstrate significant therapeutic improvement with any of these maintenance drugs. However, in similar studies done after remission induction with cyclophosphamide plus methyl-CCNU, palmO-ara-C improved therapeutic response, palmO-ara-C plus 6-thioguanine was equivocal in therapeutic response, and methotrexate or adriamycin failed to improve therapeutic response. In cross-over experiments, cyclophosphamide plus methyl-CCNU markedly improved therapeutic response after remission induction with vincristine plus prednisone, while the reverse order of treatment failed. The studies reported indicate that qualitative and quantitative methods have been developed which will allow meaningful and reliable comparisons of multidrug treatment regimens against clinically diagnosed spontaneous lymphoma in AKR mice.

[Cancer Chemother Rep Part 2, vol 4:53–72, 1974]

The AK strain of inbred mice was developed over 40 years ago by Dr. Jacob Furth (1) to establish a line of mice with a high incidence of spontaneous leukemia. He purchased mice designated arbitrarily by the symbol *A* from a commercial dealer who claimed these mice yielded many cancers, including leukemia. The original source mice were albino, random-bred, and unrelated to any other inbred strains available at that time. Furth inbred the line by brother-sister mating, selecting for breeding close relatives of leukemic mice and eliminating descendants of those remaining free of leukemia. The inbred families were designated by a second small letter added to the capital *A*. In this way families designated *Aa, Ab, Ac, Ad, Ae,* etc, were developed. After about 25 generations of brother-sister mating, about 70% of the inbred family *Ak* developed spontaneous lymphatic leukemia during the first year of life. Dr. Furth gave his subline *Ak* to many interested investigators, and the subline maintained at the Rockefeller Institute was designated *AkR*. Through common usage this subline is now identified as *AkR* or *AKR*, and these symbols are synonymous. Gross' discussion of the historical development of the AKR leukemia-lymphoma (2), including its biology and viral etiology, is detailed and valuable to anyone interested in this tumor.

Metcalf and Brumby (3) have provided a concise description of spontaneous AKR leukemia in its present-day setting:

> "AKR mice develop a higher than 90% incidence of spontaneous lymphoid leukaemia. The disease is probably initiated by the Gross leukemia virus and there is a remarkable uniformity in its gross and histological manifestations. The first neoplastic lymphoid (lymphoma) cells occur in the thymus which becomes replaced and grossly enlarged by a mass of lymphoma tissue. Subsequently there is an enlargement of all lymph nodes with moderate splenomegaly and ultimately there is an extensive infiltration by lymphoma cells of non-lymphoid organs such as the liver, kidney and ovaries. Histologically the lymphoma cells appear as very regular sheets of large lymphoid cells exhibiting high mitotic activity."

Until quite recently total in vivo destruction of even relatively small populations of transplanted murine leukemia cells was not possible with even the best drugs. However, within the past few years it has become possible to "cure" [4] advanced and clinically recognizable transplanted murine leukemias with

[4] By "cure" we mean destruction of a large enough fraction of a viable tumor cell population so that the cells surviving treatment never re-establish the clinically recognizable disease.

drugs (4,5), and we (6,7,9) and others (8) have undertaken single-drug and combination-drug chemotherapy trials against clinically diagnosed spontaneous AKR lymphoma.

METHODS OF EVALUATING RESPONSE TO CHEMOTHERAPY FOR SPONTANEOUS LEUKEMIA IN AKR MICE

We have previously described the methods we have used for establishing a clinical diagnosis of spontaneous leukemia-lymphoma in AKR mice, for bioassay for viable tumor cells surviving drug treatment in selected organs and tissues, and for evaluating drug response based on increases in median lifespan (MLS) of treated over untreated control mice and numbers of mice surviving 60 days or longer after the end of drug treatment (6,7). Briefly, these are as follows:

Diagnosis of Clinical Leukemia-Lymphoma

AKR mice between 6 and 12 months of age are palpated at weekly intervals for enlarged spleen, thymus, and superficial lymph nodes (axillary and inguinal). If all organs are significantly enlarged, a diagnosis of leukemia-lymphoma is made. If one or more of the organs (but not all) is not significantly enlarged, the mouse is set aside and the organs are repalpated in 1–4 days. Only when thymus, spleen, and lymph nodes are all enlarged is a positive diagnosis of spontaneous lymphoma made.

A. Bioassay for Viable Tumor Cells in Selected Organs and Tissues

The method of Lin and Bruce (10) as modified by us (7) is used. When used in a quantitative manner, the assay is a serial dilution method in which lymphoma cells are scored as viable when, on intravenous (iv) or intraperitoneal (ip) transplantation to young (2-month-old) syngeneic recipients, they produce splenomegaly (or leukemic death) within 28 days. The approximate number of viable lymphoma cells in an organ or tissue suspension is estimated from the successive fraction of recipients that receive tenfold dilutions of the organ or tissue being bioassayed and that develop splenomegaly using the so-called take-no take statistical method of Fisher and Yates (11).

Qualitative (all-or-none) assay for viable lymphoma cells is done similarly, except that a brei of the total organ (spleen, thymus, axillary and inguinal superficial lymph nodes) or an aliquot (liver, marrow, or blood) is implanted ip into a single young recipient AKR mouse. Spleens are examined for significant splenomegaly at death (before 35 days) or at sacrifice at 35 days. Significant splenomegaly is interpreted as indicating that one or more viable lymphoma cells was present in the entire organ or tissue (or aliquot) at the time of bioassay.

B. Therapeutic Response (Increased Lifespan) of Drug-Treated Mice

We have determined increases in lifespan (ILS) by relating the MLS of drug-treated animals to a very large historical control. The cumulative mortality of 3250 clinically diagnosed and untreated AKR mice observed in a 5-year period (1967–1972) is shown in figure 1. The MLS of these mice was 17 days, with 90% mortality by 32 days and 98.7% mortality by 60 days after diagnosis. The MLS in untreated mice shown in figure 1 is considerably longer than that reported by Goldberg et al (8) and by Kende et al (12). Because of the large variation in MLS in untreated control mice between laboratories, reliable comparisons of interlaboratory data are difficult to make. We have therefore placed major emphasis on two experimental endpoints to attempt to compare combination drug treatments reported and also to estimate the likely proximity to cell cure obtained with the treatments studied: (*a*) the percent of drug-treated mice that survived therapy and the MLS after treatment of this group, and (*b*) the number and percent of the group surviving therapy that were alive 60 days after the last drug treatment. Previously published data and interpretation thereof, based on available knowledge of the growth characteristics and population kinetics of spontaneous AKR lymphoma, indicate that one viable lymphoma cell is likely to have proliferated to the lethal number in 60 days (7,13). Therefore, it is likely that

drug-treated AKR mice with spontaneous lymphoma which are surviving 60 days after the end of drug treatment had very few, if any, viable tumor cells surviving drug therapy. Death from lymphoma beyond 60 days after therapy may be due to reinduction of another disease episode (7).

Further evidence that mice having clinically diagnosed spontaneous lymphoma and surviving 60 days after the end of drug treatment may have experienced cell cure by the drug treatment is shown in figure 2. During the course of our ongoing studies, we have implanted tenfold dilutions of monodispersed lymphoma cells from the thymus glands of AKR mice with clinically diagnosed spontaneous lymphoma iv into young AKR recipients and observed these mice until death. With those thymus cell dilution implants (10^2 or 10^3 hemocytometer-counted cells/mouse) in which some mice lived and some mice died, we can assume that each mouse implant contained, on the average, more than one viable lymphoma

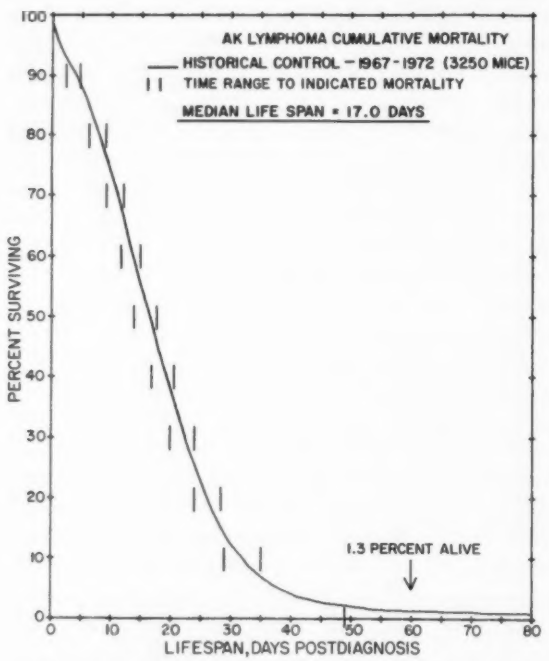
AK LYMPHOMA CUMULATIVE MORTALITY
HISTORICAL CONTROL – 1967-1972 (3250 MICE)
TIME RANGE TO INDICATED MORTALITY
MEDIAN LIFE SPAN = 17.0 DAYS
1.3 PERCENT ALIVE
PERCENT SURVIVING
LIFESPAN, DAYS POSTDIAGNOSIS

FIGURE 1.—Cumulative mortality of AKR mice after clinical diagnosis of spontaneous lymphoma, as observed in 3250 untreated control mice in a 5-year period (1967–1972).

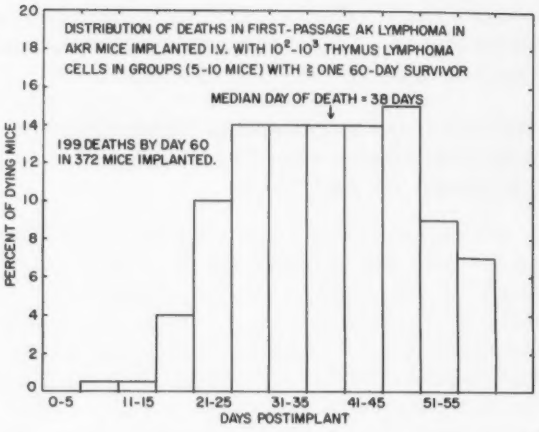
DISTRIBUTION OF DEATHS IN FIRST-PASSAGE AK LYMPHOMA IN AKR MICE IMPLANTED I.V. WITH 10^2-10^3 THYMUS LYMPHOMA CELLS IN GROUPS (5-10 MICE) WITH ≥ ONE 60-DAY SURVIVOR
MEDIAN DAY OF DEATH = 38 DAYS
199 DEATHS BY DAY 60 IN 372 MICE IMPLANTED.
PERCENT OF DYING MICE
DAYS POSTIMPLANT

FIGURE 2.—Time distribution of deaths, up to 60 days after tumor implant, in young AKR mice implanted intravenously with 10^2–10^3 (1–10 viable) hemocytometer-counted lymphoma cells from the thymus gland(s) of AKR mice with clinically diagnosed spontaneous lymphoma.

cell and fewer than ten viable lymphoma cells. (If each implant had ten cells, on the average, none would have had fewer than one cell and all implanted mice would have died [14]). In figure 2, we have shown the distribution of the deaths among the 199 of 372 mice that died in groups implanted iv with 10^2 or 10^3 hemocytometer-counted thymus cells and in which one or more mice survived for 60 days or more. The MLS of 38 days among the dying animals is consistent with all of the previously reported kinetic data (6,7,13) we have published, and it supports the contention that the individual mouse implants contained from one to ten viable lymphoma cells.

Of the 173 mice in these groups that did not die by Day 60 after implant, 45 died of leukemia and 112 survived by Day 120 after implant (at which time they were 6 months of age and subject to development of clinically recognizable spontaneous lymphoma). Thus, greater than 80% (199 of 244) of 2-month-old AKR mice dying of leukemia-lymphoma following iv implant of between one and ten viable lymphoma cells die within 60 days of implant.

These and previously published observations

(6,7) appear to support the interpretation and defend the conclusion that AKR mice with spontaneous, clinically diagnosed lymphoma that are alive 60 days after the last drug treatment probably had the total population of viable lymphoma cells at the start of treatment reduced to very small numbers, if not completely removed, by the treatment used.

C. Distribution of Viable (Transplantable) Lymphoma Cells in AKR Mice With Clinically Diagnosed Spontaneous Lymphoma

We have previously reported a wide distribution of viable tumor cells in AKR mice at diagnosis of clinically recognized spontaneous lymphoma (6,7). We have extended these observations (tables 1 and 2). These and previously published data clearly establish that when first clinically recognized the disease is quantitatively advanced and widely distributed in major lymphoid organs and also in pharmacologically protected organs (eg, brain).

CHEMOTHERAPY STUDIES

Remission Induction

A. Prednisone Plus Vincristine

The combination of an adrenocorticosteroid (usually prednisone) plus vincristine induces complete clinical remission of acute lymphocytic leukemia in nearly all children with this disease (15,16).[5] The spontaneous AKR lymphoma is both unique and useful among experimental leukemias, since a high order of remission induction can also be obtained with prednisone plus vincristine in this disease (7, 8,17).

[5] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

TABLE 1.—Quantitative bioassay for splenomegaly units (SU)* in selected organs and tissues at diagnosis of AKR mice with spontaneous lymphoma (1972)

	Spleen (whole)	Thymus (whole)	Marrow (2 femurs)	Blood (ca 1 ml)	Liver (whole)	Brain (whole)
Number of animals	14	28	14	14	14	14
Median S.U./Whole organ or tissue	7.1×10^4	1.2×10^5	2.6×10^3	2.1×10^3	3.4×10^4	5.7×10^2
Range	5.7×10^6 to 6.6×10^2	3.5×10^7 to 2.3×10^2	2.9×10^5 to 3.0×10^0	6.9×10^5 to 5.0×10^0	8.0×10^5 to $<1.1 \times 10^1$	3.3×10^4 to $<2.0 \times 10^0$

*Bioassay implants given i.p. instead of i.v. The absolute numbers of splenomegaly units are about 1 $\log_{10}$ lower than when implanted i.v. (7).

TABLE 2.—Qualitative bioassay for viable AK lymphoma cells at diagnosis

	Splenomegaly: Positive/Total, 35 Days after Tissue Implant							
	Spleen (whole)	Thymus (whole)	Marrow (2 femurs)	Nodes (axillary, inguinal)	Blood (ca 1 ml)	Liver (aliquot)	Brain (whole)	Any Tissue
	32/32	46/47	34/34	19/19	33/34	32/33	30/34	48/48
(% Negative)	(0)	(2)	(0)	(0)	(3)	(3)	(12)	(0)

TABLE 3.—Remission induction in spontaneous AK lymphoma with vincristine plus prednisone and rate of repopulation by viable cells

Treatment	Animal No.	Splenomegaly Units/Thymus: Day 1 (Day of Diagnosis)	Day 5 (24 Hr. after Last Therapy)	Day 15 (11 Days after Last Therapy)
None (Historical Control)	(52 animals)	525,000*		
Vincristine, 0.5 mg/kg, day 1 only plus Prednisone, 30 mg/kg, days 1-4	1,2		130,000	73,500
	3,4		4	294,000
	5,6		5	329,000
	7,8		27,000	6,930,000
	9,10		5	4,470,000
	11,12		3	135
	13,14		4	2,220
	15,16		5	3,100
	17,18		415	275,000
	19,20		346	1,680
	Median:	525,000	ca 5	174,250

*Median

TABLE 4.—Remission induction in spontaneous AK lymphoma with vincristine plus prednisone: spleen and thymus weights following remission induction

Treatment	Animal No.	Thymus and Spleen Weights (mg) of the Treated Animals: Day 1 Thymus	Day 1 Spleen	Day 5 Thymus	Day 5 Spleen	Day 15 Thymus	Day 15 Spleen
None (Historical Control)	(120 animals)	628*	510*				
Vincristine, 0.5 mg/kg, day 1 only plus Prednisone, 30 mg/kg, days 1-4	1,2			85	240	425	225
	3,4			110	30	170	460
	5,6			90	80	190	575
	7,8			160	170	180	300
	9,10			90	40	130	395
	11,12			80	30	70	510
	13,14			100	40	290	350
	15,16			110	20	90	60
	17,18			60	35	320	310
	19,20			50	95	110	220
	Median:	628	510	90	40	175	330

*Median

The data in tables 3 and 4 illustrate the remission induction by prednisone plus vincristine as demonstrated by reduction in splenomegaly units (viable lymphoma cells) in the thymus (table 3) and weight reduction of spleen and thymus (table 4).

By Day 5 (24 hours after the last treatment) an approximate 5-$\log_{10}$ reduction of viable lymphoma cells in the thymus was observed, but 10 days later (Day 15) viable tumor cells in the thymus glands were back to approximately the same level as at diagnosis and before remission induction (table 3). Twenty-four hours after the last dose of prednisone (Day 5) nearly all of the spleen and thymus weights were reduced to normal, but by Day 15 marked enlargement of these organs had reoccurred (table 4), accompanying the regrowth of viable lymphoma cells (table 3).

TABLE 5.—Reduction of viable AKR lymphoma cells in the thymus by vincristine, prednisone, or vincristine plus prednisone: $\log_{10}$ kill by bioassay (SU*/thymus) 24 hours after end of therapy

Therapy (mg/kg/dose)	Log Kill		
	Vincristine Alone	Prednisone Alone	Vincristine + Prednisone
Vincristine, 2.0, day 1 only + Prednisone, 30, days 1-4		1.5	5.8
Vincristine, 1.0, day 1 only + Prednisone, 30, days 1-4	1.5	1.5	5.5
Vincristine, 0.75, day 1 only + Prednisone, 30, days 1-4		1.5	5.2
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4		1.5	5.0
Vincristine, 0.5, days 1 and 11 + Prednisone, 30, days 1-4 and 11-14			4.7
Vincristine, 0.25, days 1 and 5 + Prednisone, 30, days 1 and 5			3.9

*S.U. = Splenomegaly Units (10)

In table 5 are listed $\log_{10}$ cell kill of viable lymphoma cells in the thymus gland by vincristine or prednisone and by the two drugs in combination as determined by splenomegaly unit (SU) assay (10). Several important points are apparent from these data:

1. The two drugs in combination show significantly greater-than-additive lymphoma cell kill.
2. Drug combination synergism is apparent with vincristine over a range of doses from 2.0 to 0.25 mg/kg.
3. Remission reinduction can be obtained by a second course of vincristine plus prednisone (*see* tables 3 and 5).[6]

B. Other Remission-Inducing Drug Combinations

We have previously reported that cyclophosphamide plus an active nitrosourea (eg, methyl-CCNU) and arabinosylcytosine (ara-C) plus cyclophosphamide are among the most therapeutically effective two-drug combinations against spontaneous AKR lymphoma (7). Based on SU assays for viable lymphoma cells in the thymus glands of drug-treated mice, we have shown that greater lymphoma cell kill can be obtained when cyclophosphamide plus BCNU or ara-C is used (tables 6 and 7) than when any one of the three drugs is used alone.

We have used (*a*) prednisone plus vincristine, (*b*) cyclophosphamide plus methyl-CCNU, and (*c*) cyclophosphamide plus ara-C for remission induction, and the relative activities of these three combinations, based on lifespan and 60-day posttherapy survivors, are summarized in table 8. Approximately the same fraction of treated animals survived the remission-induction regimen with all three-drug combinations, but the combination of vincristine plus prednisone was much less effective in increasing the MLS or achieving long-term survivors (60 days posttherapy) than the other two-drug combinations.

This is somewhat confusing, since the three combinations of two drugs have about the same activity against AKR lymphoma cells in the thymus glands of clinically diagnosed mice with spontaneous lymphoma, based on $\log_{10}$ cell kill (tables 5–7). However, it may be due to the fact that drug combinations *b* and *c* above have a nitrosourea or ara-C which crosses the so-called blood-brain barrier and kills lymphoma cells in the CNS, and at diagnosis AKR mice with spontaneous lymphoma have a high incidence of lymphoma cells in the brain (table 2). Prednisone enters the cere-

[6] Vincristine, Days 1 and 11, and prednisone, Days 1–4 and 11–14.

TABLE 6.—Reduction of viable AKR lymphoma cells in the thymus by cyclophosphamide, BCNU, or cyclophosphamide plus BCNU: $\log_{10}$ kill by bioassay (SU*/thymus) 24 hours after end of therapy

Therapy (mg/kg/dose)	Log Kill		
	Cyclophosphamide Alone	BCNU Alone	Cyclophosphamide + BCNU
Cyclophosphamide, 312, day 1 only + BCNU, 41, day 1 only	4.4	2.3-3.9	4.7
Cyclophosphamide, 208, day 1 only + BCNU, 41, day 1 only		2.3-3.9	4.7
Cyclophosphamide, 208, day 1 only + BCNU, 20.5, day 1 only		1.2	4.4
Cyclophosphamide, 208, day 1 only + BCNU, 10.25, day 1 only		0.9	4.7
Cyclophosphamide, 156, day 1 only + BCNU, 27, day 1 only	3.8		4.7
Cyclophosphamide, 78, day 1 only + BCNU, 27, day 1 only	3.2		4.7
Cyclophosphamide, 50, day 1 only + BCNU, 15, day 1 only	2.3		4.0
Cyclophosphamide, 39, day 1 only + BCNU, 27, day 1 only	3.1		4.2
Cyclophosphamide, 37, day 1 only + BCNU, 10, day 1 only		0.9	4.2

*S.U. = Splenomegaly Units (10)

TABLE 7.—Reduction of viable AKR lymphoma cells in the thymus by cyclophosphamide, ara-C, or cyclophosphamide plus ara-C: $\log_{10}$ kill by bioassay (SU*/thymus) 24 hours after end of therapy

Therapy (mg/kg/dose)	Cyclophosphamide Alone	Arabinosylcytosine Alone**	Cyclophosphamide + Arabinosylcytosine
Cyclophosphamide, 150, day 1 only + Ara-C, 7.5, Q3H x 3, Days 4-10	1.9-4.3	0.1	5.2

*S.U. = Splenomegaly Units (10)

**Q3H x 3, Day 1 only

TABLE 8.—Three remission-inducing two-drug combinations

Remission-inducing Therapy	% Mice Surviving Therapy	Median Life Span (Days) of Mice Surviving Therapy	Surviving 60 Days after Therapy (%)
Vincristine + Prednisone	83	23.5	7/389 (2)
Cyclophosphamide + MeCCNU	77	32.8	53/356 (15)
Ara-C + Cyclophosphamide	85	34.0	61/382 (16)

TABLE 9.—Effect of repeated courses and reversal of the order of drug administration on therapeutic response of AKR mice with spontaneous lymphoma: palmO-ara-C→ cyclophosphamide, and cyclophosphamide→ ara-C

Therapy (mg/kg/dose)	Last Day of Therapy	Median Life Span (Days) of Mice Surviving Therapy	Survivors at End of Therapy/ Total (%)	Survivors 60 Days after Therapy (%)
PalmO-ara-C, 100, days 1 & 15 + Cyclophosphamide, 100, days 3 & 17	17	44.5	26/36 (72)	9/26 (35)
PalmO-ara-C, 100, days 1 & 15 + Cyclophosphamide, 150, days 3 & 17	17	44.5	33/40 (83)	8/33 (24)
PalmO-ara-C, 150, day 1 only + Cyclophosphamide, 150, day 3 only	3	37.0	35/35 (100)	4/35 (11)
PalmO-ara-C, 30, days 1-5 + Cyclophosphamide, 150, day 7 only	7	32.0	28/31 (90)	4/28 (14)
PalmO-ara-C, 150, day 1 only + Cyclophosphamide, 150, day 5 only	5	31.0	30/32 (94)	3/30 (10)
PalmO-ara-C, 100, days 1, 15, 29 + Cyclophosphamide, 100, days 3, 17, 31	31	30.0	30/37 (81)	6/30 (20)
			182/211 (86)	34/182 (19) or 16% of all
Cyclophosphamide, 150, days 1, 13 + Ara-C, 7.5, Q3H x 3, Days 4-10, 16-22	22	38.5	72/101 (71)	18/72 (25)
Cyclophosphamide, 100, days 1, 13 + Ara-C, 7.5, Q3H x 3, days 4-10, 16-22	22	30.0	73/96 (76)	6/73 (8)
			145/197 (74)	24/145 (17) or 12% of all

brospinal fluid in therapeutically effective levels following oral or parenteral administration in man and thus is effective in controlling meningeal leukemic infiltrates (16), but the combination of vincristine plus prednisone may not be effective against meningeal leukemic infiltrates in the AKR mouse.

C. Repeated Courses of Ara-C or PalmO-ara-C, a Depot Form of Ara-C, Plus Cyclophosphamide and the Influence of Drug Sequence

Sequential courses of ara-C (or palmO-ara-C) plus cyclophosphamide and reversal of the order of sequential drug administration have been studied. The results of these studies are listed in table 9. In the first experiment listed in table 9, 26 of 36 AKR mice with clinically diagnosed spontaneous lymphoma, treated as shown, were alive on Day 17 (72% survived drug treatment), and 35% of these mice (nine of 26) were alive 60 days after the last drug treatment. The various treatment regimens have been listed in table 9 in order of decreasing MLS of those mice alive at the end of drug treatment.

The therapeutic responses listed in table 9 are very impressive. For example, in the first two treatments listed, 72% or more of treated mice were alive on Day 17 (which is the median day of death of untreated controls; *see* fig 1), and the MLS after Day 17 was an additional 44.5 days, with 24%–35% of those surviving drug treatment still alive 60 days later (Day 77 after diagnosis). These data indicate the likelihood that, at least in these animals, the

number of viable lymphoma cells surviving drug therapy was small. It appears that a better therapeutic effect may be obtained if cyclophosphamide follows rather than precedes ara-C in these sequential schedules.

D. Repeated Courses of Cyclophosphamide Plus Methyl-CCNU

Several studies in which two courses of cyclophosphamide plus methyl-CCNU were given simultaneously have been done, and the results of these studies are listed in table 10. It appears that repeated courses of cyclophosphamide plus methyl-CCNU provided additional therapeutic gain over a single course, since the MLS of survivors of a single course of therapy was 32.8 days (table 8), and the MLS in survivors of two courses ranged from 33.5 to 40.0 days (table 10).

FOUR-DRUG COMBINATION CHEMOTHERAPY FOR SPONTANEOUS LYMPHOMA IN AKR MICE

Remission Induction With Prednisone Plus Vincristine Followed by Consolidation Treatment With Other Drug Combinations

A. Cyclophosphamide→ Ara-C (or PalmO-ara-C)

A number of experiments were carried out in which remission was induced by vincristine plus prednisone followed by cyclophosphamide and ara-C (or palmO-ara-C). Results of these studies are listed in table 11. The first experiment listed in table 11 is clearly the best therapeutic response we have seen with spontaneous AKR lymphoma. The MLS of the 21 of 33 (64%) mice surviving treatment was 51.5 days after the end of treatment, and nine of these 21 mice (43%) were alive 60 days after the end of therapy.

Qualitative bioassays for viable lymphoma cells in a variety of organs and tissues were made in mice treated with this best therapy (vincristine + prednisone→ cyclophosphamide → palmO-ara-C [*see* first and best results, table 11]). Bioassays were made 24 hours after each segment of the four-drug sequence of treatment and again at 1 day, 2 weeks, 4 weeks, and 6 weeks after the end of treatment. Results of these bioassays are shown in table 12. Viable lymphoma cells were present in most organs and tissues assayed and in one or more tissues of all mice assayed 24 hours after the first dose of vincristine and prednisone (Day 2). Following the remission-induction course of vincristine plus prednisone (Day 5), a number of organs were free of viable lymphoma cells, but seven of eight animals bioassayed still had recoverable lymphoma cells in one or more organs and/or tissues assayed. Twenty-four hours after the single dose of cyclophosphamide (Day 6) and after the course of palmO-ara-C (Day 13), the numbers of organs and tissues free of viable lymphoma cells were lowest. This relatively low incidence of organs and tissues containing lymphoma cells persisted for an additional 2 weeks and then rose again. These data are similar to those listed in table 11 suggesting that the four-drug therapy reduces the viable lymphoma cell population at the end of therapy to small numbers, but the lack of large numbers of long-term survivors indicates that total cell cure in most animals may not have been achieved.

B. Ara-C (or PalmO-ara-C)→ Cyclophosphamide

A set of experiments similar to those described in the above section was done, differing only in that the ara-C preceded the cyclophosphamide in the sequential consolidation treatment. Results of these experiments are summarized in table 13.

Seventy-nine percent of all treated mice survived the various treatment schedules (9–19 days), and very significant duration of life in these treatment survivors was observed (MLS of 28–49.5 days). Large single doses of palmO-ara-C appeared to be better than smaller repeated doses. Delay in starting small multiple daily doses of ara-C was less effective than starting ara-C shortly after finishing remission-induction therapy. No apparent advantage was noted for the ara-C→ cyclophosphamide sequence over the cyclophosphamide→ ara-C sequence (*see* section A above and table 11).

TABLE 10.—Therapeutic response of AKR mice with spontaneous lymphoma to repeated courses of cyclophosphamide plus methyl-CCNU

Therapy (mg/kg/dose)	Last Day of Therapy	Median Life Span (Days) of Mice Surviving Therapy	Survivors at End of Therapy/ Total (%)	Survivors 60 Days after Therapy (%)
Cyclophosphamide, 100, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	40.0	55/68 (81)	14/55 (25)
Cyclophosphamide, 100, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	39.0	71/128 (55)	15/71 (21)
Cyclophosphamide, 67, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	38.0	27/30 (90)	2/27 (7)
Cyclophosphamide, 67, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	34.5	22/30 (73)	5/22 (23)
Cyclophosphamide, 100, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	34.5	49/75 (65)	7/49 (14)
Cyclophosphamide, 100, days 1 & 10 + MeCCNU, 16, days 1 & 10	10	33.5	27/40 (68)	0/27 (0)
			251/371 (68)	43/251 (17) or 12% of all

C. PalmO-ara-C→ CCNU or Methyl-CCNU, and CCNU→ PalmO-ara-C

Similar experiments were done in which palmO-ara-C→ CCNU or methyl-CCNU, and CCNU→ palmO-ara-C treatment was given following remission induction with prednisone plus vincristine. Results of these experiments are summarized in table 14. Here, small daily doses of palmO-ara-C appeared to be more effective than a single larger dose, and the sequence of CCNU→palmO-ara-C failed to give any survivors 60 days after the end of treatment. It is apparent that palmO-ara-C plus CCNU or methyl-CCNU effectively extends the MLS and long-term survivor rates of AKR mice in remission induced by vincristine plus prednisone.

MAINTENANCE THERAPY WITH METHOTREXATE, ADRIAMYCIN, PALMO-ARA-C, OR PALMO-ARA-C PLUS 6-THIOGUANINE FOLLOWING REMISSION INDUCTION WITH EITHER VINCRISTINE PLUS PREDNISONE OR CYCLOPHOSPHAMIDE PLUS METHYL-CCNU

Since modern drug treatment of acute lymphatic leukemia in children usually involves remission induction with vincristine plus prednisone followed by maintenance therapy with one or more other antileukemic drugs (16), we carried out some experiments to compare therapeutic response to maintenance therapy with methotrexate, adriamycin, palmO-ara-C, or palmO-ara-C plus 6-thioguanine following remission induction with either prednisone plus vincristine or cyclophosphamide plus methyl-CCNU. The comparative activity of the two remission-inducing treatments (prednisone plus vincristine and cyclophosphamide plus methyl-CCNU), as indicated by the fraction surviving remission-induction therapy and the fraction achieving complete clinical remission, is shown in table 15.

In ten separate experiments in which 80–125 AKR mice with clinically diagnosed spontaneous lymphoma were treated with vincristine plus prednisone, 83% (range, 79%–90%) survived treatment, and 5 days after the last treatment 47% (range, 14%–90%) of those surviving treatment were in complete clinical remission, as determined by palpation of spleen, thymus, and peripheral lymph nodes. These results are similar to those reported by Goldberg et al (8).

TABLE 11.—Four-drug combination treatment of spontaneous lymphoma in AKR mice: remission induction → consolidation and vincristine plus prednisone (or dexamethasone) → cyclophosphamide plus ara-C (or palmO-ara-C)

Therapy (mg/kg/dose)	Last Day of Therapy	Median Life Span (Days) of Mice Surviving Therapy	Survivors at End of Therapy/ Total (%)	Survivors 60 Days after Therapy (%)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 Cyclophosphamide, 150, day 5 only + PalmO-ara-C, 25, days 8-12	12	51.5	21/33 (64)	9/21 (43)
Vincristine, 1.0, day 1 only + Prednisone, 30, days 1-4 Cyclophosphamide, 150, day 6 only + Ara-C, 7.5, Q3H x 4, days 9-14	14	45.0	10/22 (45)	3/10 (30)
Vincristine, 0.5, day 1 only + Dexamethasone, 4.5, days 1-4 Cyclophosphamide, 50, day 1 only + PalmO-ara-C, 50, day 1 only	4	36.5	25/30 (83)	5/25 (20)
Vincristine, 0.5, days 1, 5, 9 + Prednisone, 13, days 1, 5, 9 Cyclophosphamide, 100, day 11 only + Ara-C, 7.5, Q3H x 3, days 14-20	20	30.5	61/88 (69)	9/61 (15)
Vincristine, 0.5, day 1 only + Dexamethasone, 4.5, days 1-4 Cyclophosphamide, 100, day 1 only + PalmO-ara-C, 100, day 1 only	4	29.5	25/32 (78)	5/25 (20)
			142/205 (69)	31/142 (22) or 15% of all

In four separate experiments in which 77–139 similar mice were treated with cyclophosphamide plus methyl-CCNU, 77% (range, 71%–89%) survived treatment, and 4 days after the last treatment 84% (range, 65%–94%) of those surviving treatment were in complete clinical remission. About the same fraction of mice treated with each remission-inducing drug combination survived the treatment, but a higher incidence of complete

TABLE 12.—Qualitative bioassay for viable AK cells during and following remission induction with prednisone plus vincristine followed by treatment with cyclophosphamide and palmO-ara-C

Therapy (mg/kg/dose)	Bioassay Time	Splenomegaly: Positive/Total, 35 Days after Tissue Implant Spleen (whole)	Thymus (whole)	Marrow (2 femurs)	Nodes (axillary, inguinal)	Blood (ca 1 ml)	Liver (aliquot)	Brain (whole)	Any Tissue
Vincristine, 0.5, day 1 only Prednisone, 30, days 1-4 Cyclophosphamide, 150, day 5 only PalmO-ara-C, 25, days 8-12	Day 2	6/8	8/8	6/8	6/7	6/8	7/8	4/8	8/8
	Day 5	5/7	5/8	4/8	2/8	1/8	4/8	3/8	7/8
	Day 6	5/8	3/7	3/8	3/7	3/8	6/8	4/8	6/8
	Day 13	4/7	2/7	1/8	2/7	1/8	4/8	2/8	8/8
	Day 27	3/6	3/7	4/8	3/7	3/8	3/7	3/8	6/8
	Day 42	8/8	8/10	7/9	7/9	6/9	8/8	6/7	10/10
	Day 57	2/2	2/2	2/2	2/2	1/2	2/2	1/2	2/2

TABLE 13.—Four-drug combination treatment of spontaneous lymphoma in AKR mice: remission induction→ consolidation and vincristine plus prednisone (or dexamethasone)→ ara-C (or palmO-ara-C) plus cyclophosphamide

Therapy (mg/kg/dose)	Last day of Therapy	Median Life Span (Days) of Mice Surviving Therapy	Survivors at End of Therapy/ Total (%)	Survivors 60 Days after Therapy (%)
Vincristine, 1.0, day 1 only + Prednisone, 30, days 1-4 Ara-C, 7.5, Q3H x 4, days 5-10 + Cyclophosphamide, 150, day 13 only	13	49.5	9/20 (45)	3/9 (33)
Vincristine, 0.5, day 1 only + Dexamethasone, 4.5, days 1-4 PalmO-ara-C, 150, day 7 only +Cyclophosphamide, 150, day 11 only	11	40.5	23/31 (74)	7/23 (30)
Vincristine, 1.0, day 1 only + Prednisone, 30, days 1-4 Ara-C, 7.5, Q3H x 4, days 11-16 + Cyclophosphamide, 150, day 19 only	19	40.0	13/18 (72)	0/13 (0)
Vincristine, 0.25, days 1 & 5 + Prednisone, 30, days 1 & 5 PalmO-ara-C, 100, day 8 only + Cyclophosphamide, 150, day 12 only	12	38.5	91/100 (91)	14/91 (15)
Vincristine, 0.5, day 1 only + Dexamethasone, 4.5, days 1-4 PalmO-ara-C, 150, day 7 only + Cyclophosphamide, 150, day 9 only	9	37.5	25/31 (81)	9/25 (36)
Vincristine, 0.25, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 100, day 7 only + Cyclophosphamide, 150, day 11 only	11	34.0	40/57 (70)	8/40 (20)
Vincristine, 0.25, day 1 only + Dexamethasone, 4.5, days 1-4 PalmO-ara-C, 100, day 7 only + Cyclophosphamide, 150, day 11 only	11	32.5	79/98 (81)	11/79 (14)
Vincristine, 0.25, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 100, day 7 only + Cyclophosphamide, 100, day 11 only	11	32.0	41/43 (95)	5/41 (12)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 25, days 5-8 + Cyclophosphamide, 150, day 12 only	12	28.0	17/31 (55)	0/17 (0)
			338/429 (79)	57/338 (17) or 13% of all

clinical remissions was seen in survivors among those treated with cyclophosphamide plus methyl-CCNU.

We carried out a series of experiments in which groups of between 80 and 197 AKR mice with clinically diagnosed spontaneous lymphoma were treated with either vincristine plus prednisone or cyclophosphamide plus methyl-CCNU, as described above. At the time of the start of maintenance therapy, the survivors of the remission-induction therapy were examined for complete clinical remission of the spontaneous lymphoma, but they were randomly distributed into two approximately equal groups without regard to clinical status. Maintenance therapy with methotrexate, adriamycin, palmO-ara-C, or palmO-ara-C plus 6-

TABLE 14.—Four-drug combination treatment of spontaneous lymphoma in AKR mice: remission induction→ consolidation and vincristine plus prednisone→ palmO-ara-C plus CCNU or methyl-CCNU

Therapy (mg/kg/dose)	Last Day of Therapy	Median Life Span (Days) of Mice Surviving Therapy	Survivors at End of Therapy/ Total (%)	Survivors 60 Days after Therapy (%)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 25, days 5-9 + CCNU, 25, day 12 only	12	46.5	18/31 (58)	7/18 (39)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 CCNU, 25, day 5 only + PalmO-ara-C, 25, days 8-12	12	40.0	19/30 (63)	0/19 (0)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 25, days 5-9 + CCNU, 25, day 12 only	12	35.5	23/34 (68)	6/23 (26)
Vincristine, 0.5, day 1 only + Prednisone, 30, days 1-4 PalmO-ara-C, 75, day 7 only + MeCCNU, 27, day 10 only	10	31.0	18/30 (60)	2/18 (11)
			78/125 (62)	15/78 (19) or 12% of all

thioguanine was given to one group, and the other group was held as a control on the maintenance treatment group. Results of these studies are summarized in tables 16–19.

A. Maintenance Therapy With Methotrexate

No significant increase in MLS was obtained by maintenance therapy with methotrexate at the doses and schedules used in either group. The internal variables in experiments such as these make reliable comparisons of responses difficult, if not impossible. For example, the fraction surviving either remission-induction therapy was similar, but the percent in complete remission following cyclophosphamide plus methyl-CCNU treatment was about double that with vincristine plus prednisone. The numbers of viable lymphoma cells present in each mouse surviving remission-inducing therapy with cyclophosphamide plus methyl-CCNU were probably very small, since (*a*) over 90% of the mice were in complete clinical remission, (*b*) the MLS was 41 days after the last treatment and five of 39 mice were alive 60 days after the last treatment, and (*c*) maintenance therapy with methotrexate at low doses (0.15 mg/kg/day × 7 in each course) was probably toxic, since it reduced MLS as compared to untreated controls, indicating likely residual toxicity from the cyclophosphamide plus methyl-CCNU remission-induction treatment. Observations such as these impress upon us the great difficulties in adequately controlling multiple-drug combination chemotherapy trials and greatly increase our respect for the even greater similar problems facing the clinician treating human patients with drugs.

B. Maintenance Therapy With Adriamycin

Studies similar to those described with methotrexate were done with adriamycin. Results

TABLE 15.—Comparative remission induction of spontaneous lymphoma in AKR mice with vincristine plus prednisone and cyclophosphamide plus methyl-CCNU

Vincristine plus Prednisone Vincristine, 0.25 mg/kg, IP, days 1 & 5; Prednisone, 30 mg/kg, SC, days 1 & 5			Cyclophosphamide plus MeCCNU Cyclophosphamide, 100 mg/kg, IP, days 1 & 10; MeCCNU, 16 mg/kg, IP, days 1 & 10		
Number Mice Started	Number Surviving Induction Therapy (%)	Number in Complete Remission*(%)	Number Mice Started	Number Surviving Induction Therapy (%)	Number in Complete Remission*(%)
115	97 (84)	46 (47)	107	77 (72)	72 (94)
121	100 (83)	54 (54)	125	99 (79)	82 (83)
110	93 (85)	48 (52)	197	139 (71)	131 (94)
80	72 (90)	27 (38)	122	109 (89)	71 (65)
124	98 (79)	28 (29)	551	424 (77)	356 (84)
115	93 (81)	31 (33)			
104	85 (82)	12 (14)			
125	102 (82)	64 (63)			
100	88 (88)	79 (90)			
994	828 (83)	389 (47)			

*Spleen, thymus, and peripheral lymph nodes of normal size as determined by palpation, 4-5 days after last treatment.

of these studies are summarized in table 17. No significant activity was observed with maintenance treatment with adriamycin. However, these results are subject to the same reservations and qualifications discussed in section A above.

C. Maintenance Therapy With PalmO-ara-C

Results of these studies are summarized in table 18. In these experiments maintenance therapy with palmO-ara-C did not significantly alter the lifespan following remission-induction therapy with vincristine plus prednisone, but it did significantly increase the MLS and long-range survival following cyclophosphamide plus methyl-CCNU remission-induction therapy. As commonly seen, cyclophosphamide plus methyl-CCNU resulted in a high complete clinical remission rate (94%) in mice surviving remission-induction therapy. On Day 70 after diagnosis (60 days after the end of remission-induction therapy), 13 of the 71 mice in the control group were still alive. At that time 21 of the 69 mice in the palmO-ara-C maintenance group were still alive, and the MLS of this group of 69 mice was increased to 56.5 days after the end of remission-induction treatment (Day 10).

D. Maintenance Therapy With PalmO-ara-C Plus 6-Thioguanine

Since ara-C plus 6-thioguanine is one of the treatment combinations of choice for acute leukemia in adults (18), and since it is a therapeutically potentiating drug combination against L1210 leukemia (19), we used this combination as maintenance therapy in AKR lymphoma. Results of these studies are listed in table 19. No significant effect was seen with this maintenance treatment following remission-induction therapy with vincristine plus prednisone. However, a low order (33%–42%) of complete clinical remission was present at the start of maintenance therapy. In these ex-

TABLE 16.—Comparison of maintenance therapy with methotrexate following remission-induction treatment with either vincristine plus prednisone or cyclophosphamide plus methyl-CCNU: remission induction (RI) → maintenance (M) therapy

Therapy (mg/kg/dose)	Alive at Start of Maintenance (%)	Maintenance Therapy Groups No. of Mice (% in Remission*)	Median Life Span (Days) From Start of Maintenance ( )**
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5	97/115(84)	48 Control*** (50)	23.0
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 + M { Methotrexate, 0.15, QD x 7, Q14D x 5, from day 10		49 (45)	25.0
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10	77/107(72)	39 Control*** (90)	41.0 (5/39)
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10 + M { Methotrexate, 0.15, QD x 7, Q14D x 6, from day 14		38 (97)	34.0

*Percent in complete clinical remission at start of maintenance therapy.
**( )Survivors/Total, 60 days after last therapy.
***No further treatment.

TABLE 17.—Comparison of maintenance therapy with adriamycin following remission-induction treatment with either vincristine plus prednisone or cyclophosphamide plus methyl-CCNU: remission induction (RI) → maintenance (M) therapy

Therapy (mg/kg/dose)	Alive at Start of Maintenance (%)	Maintenance Therapy Groups No. of Mice (% in Remission*)	Median Life Span (Days) From Start of Maintenance ( )**
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5	101/121 (83)	51 Control*** (58)	22.5 (3/51)
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 + M { Adriamycin, 0.75, QD x 7, Q14D x 3, from day 10		50 (50)	23.0
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10	99/125 (79)	49 Control*** (82)	43.5 (5/49)
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10 + M { Adriamycin, 0.75, QD x 7, Q14D x 6, from day 14		50 (84)	39.0 (1/50)

*Percent in complete clinical remission at start of maintenance therapy.
**( ) Survivors/Total, 60 days after last therapy.
***No further treatment.

TABLE 18.—Comparison of maintenance therapy with palmO-ara-C following remission-induction treatment with either vincristine plus prednisone or cyclophosphamide plus methyl-CCNU: remission induction (RI) → maintenance (M) therapy

Therapy (mg/kg/dose)	Alive at Start of Maintenance (%)	Maintenance Therapy Groups No. of Mice (% in Remission*)	Median Life Span (Days) From Start of Maintenance ( )**
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5	93/110 (85)	46 Control*** (46)	24.5
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 + M { PalmO-ara-C, 5, QD x 7, Q14D x 6, from day 10		47 (57)	26.5
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10	140/197 (71)	71 Control*** (94)	49.0 (13/71 on day 70; **** 8/71 on day 87)
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10 + M { PalmO-ara-C, 5, QD x 7, Q14D x 13, from day 14		69 (94)	56.5 (21/69 on day 70; 14/69 on day 87; 7/69 on day 127; all dead day 194)

*Percent in complete clinical remission at start of maintenance therapy.
**( ) Survivors/Total.
***No further treatment.
****Sacrificed for bioassay on day 87; tissues bioassayed as in Table 12; 7/8 positive in any tissue.

periments maintenance therapy with palmO-ara-C plus 6-thioguanine following cyclophosphamide plus methyl-CCNU was not as good as with palmO-ara-C alone (table 18). The MLS was shorter in mice receiving maintenance therapy than in the controls, and the survivors in both groups at 70 days (60 days after last remission-induction treatment with cyclophosphamide plus methyl-CCNU) were the same—13 of 55 and 13 of 54. This may have been because the fraction of treated mice in complete remission at the start of maintenance therapy was lower than in the experiment in which maintenance with palmO-ara-C alone was used.

E. Vincristine Plus Prednisone→ Cyclophosphamide Plus Methyl-CCNU, and Cyclophosphamide Plus Methyl-CCNU→ Vincristine Plus Prednisone

An experiment was carried out comparing the effects of the two remission-inducing drug combinations in cross-over treatment. Remission induction with vincristine plus prednisone was followed by treatment with cyclophosphamide plus methyl-CCNU, and remission induction with cyclophosphamide plus methyl-CCNU was followed by treatment with vincristine plus prednisone. Results of these studies are summarized in table 20. In this experiment a very low complete clinical remission rate following vincristine plus prednisone was observed (14%). However, maintenance treatment with cyclophosphamide plus methyl-CCNU more than doubled the MLS of mice surviving remission-induction treatment (22.5–46.5 days after start of maintenance [Day 10]).

No significant effect of vincristine plus prednisone on lifespan was seen in mice treated with cyclophosphamide plus methyl-CCNU as remission-induction therapy.

In a single experiment (table 21) we carried out qualitative bioassays for viable lymphoma cells in a number of organs and tissues of mice 24 hours after the last treatment, in which remission-induction therapy

TABLE 19.—Comparison of maintenance therapy with palmO-ara-C plus 6-thioguanine following remission-induction treatment with either vincristine plus prednisone or cyclophosphamide plus methyl-CCNU: remission induction (RI) → maintenance (M) therapy

Therapy (mg/kg/dose)	Alive at Start of Maintenance (%)	Maintenance Therapy Groups No. of Mice (% in Remission*)	Median Life Span (Days) From Start of Maintenance ( )**
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5	72/80 (90)	36 Control*** (33)	22.0 (1/36)
RI { Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 + M { PalmO-ara-C, 5, QDx7, Q14Dx4, from day 10 6-Thioguanine, 0.5, QDx7, Q14Dx4, from day 10		36 (42)	25.0
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10	109/122 (89)	55 Control*** (69)	50.0 (13/55 on day 70; ****11/55 on day 78)
RI { Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10 + M { PalmO-ara-C, 5, QDx7, Q14Dx6, from day 14 6-Thioguanine, 0.5, QDx7, Q14Dx6, from day 14		54 (61)	42.5 (13/54 on day 70; 10/54 on day 78; 5/54 on day 103; 1 survivor bioassayed and positive on day 157)

*Percent in complete clinical remission at start of maintenance therapy.
**( ) Survivors/Total, 60 days after last therapy.
***No further treatment.
****Sacrificed for bioassay on day 78; tissues bioassayed as in Table 12; 10/11 positive in any tissue.

with vincristine plus prednisone was followed by simultaneous treatment with cyclophosphamide plus methyl-CCNU on Days 10 and 20 (the same drug treatment shown in table 20). Qualitative bioassay for viable lymphoma cells indicated that many organs were cleared of recoverable viable lymphoma cells, and in only 50% (five of ten mice) were they recovered from any organ or tissue, again indicating that treatment with this best four-drug sequential treatment probably approaches cure of the cellular form of this spontaneous leukemia-lymphoma in the AKR mouse.

We have previously reported a 45% (nine of 20 mice) clearance rate of recoverable viable lymphoma cells in all bioassayed organs and tissues following treatment with cyclophosphamide plus ara-C (7).

Goldberg et al have carried out comparative maintenance therapy experiments following remission induction with vincristine plus prednisone (8). They clinically staged their mice following remission-induction therapy with vincristine plus prednisone and separated those in clinical remission from those retaining clinical evidence of disease on day 7, considering only those in complete remission in their maintenance treatment studies. They also continued maintenance therapy to death. As a result, there was a wide range of total drug(s) given to different mice in each treatment group, and they did not report their results in terms of survivors at some point in time after the start, or end, of maintenance therapy. Since their MLS from the end of remission induction was about 13 days and the greatest increase in MLS they reported was 267%, or about 45 days, with cyclophosphamide, it appears that we may have observed a somewhat greater therapeutic response with palmO-ara-C plus cyclophosphamide when used alone. We observed a 44.5-day MLS among those surviving drug treatment. Nine of 36 (25%) mice were alive 60 days after therapy, and they were from an original group with clinically diagnosed AKR lymphoma without any selection during treatment for purposes of clinical staging (table 9). Very similar results were

TABLE 20.—Comparison of maintenance therapy with cyclophosphamide plus methyl-CCNU following remission-induction treatment with vincristine plus prednisone and with vincristine plus prednisone following remission-induction treatment with cyclophosphamide plus methyl-CCNU: remission induction (RI) → maintenance (M) therapy

Therapy (mg/kg/dose)	Alive at Start of Maintenance (%)	Maintenance Therapy Groups No. of Mice (% in Remission*)	Median Life Span (Days) From Start of Maintenance ( )**
RI{Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5	85/104 (82)	43 Control*** (14)	22.5
RI{Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 + M {Cyclophosphamide, 100, days 10 and 20 MeCCNU, 16, days 10 and 20		42 (14)	46.5 (4/42)
RI{Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10	102/125 (82)	51 Control*** (67)	43.5 (3/51)
RI{Cyclophosphamide, 100, days 1 and 10 MeCCNU, 16, days 1 and 10 + M {Vincristine, 0.25, days 15 and 19 Prednisone, 30, days 15 and 19		51 (59)	38.5 (2/51)

*Percent in complete clinical remission at start of maintenance therapy.
**( ) Survivors/Total, 60 days after last therapy.
***No further treatment.

obtained in similar groups, similarly evaluated, that were treated with cyclophosphamide plus palmO-ara-C following remission-induction treatment with vincristine plus prednisone (table 11).

Like Goldberg et al (8), we saw no clearly significant activity of methotrexate or palmO-ara-C for maintenance therapy (tables 16 and 18) following remission induction with vincristine plus prednisone, but activity was noted for palmO-ara-C following remission-induction treatment with cyclophosphamide plus methyl-CCNU (table 18). They observed significant activity with cyclophosphamide, similar

TABLE 21.—Qualitative bioassay for viable AK lymphoma cells in mice with clinically diagnosed spontaneous lymphoma during and following remission-induction treatment with prednisone plus vincristine followed by cyclophosphamide and methyl-CCNU

		Splenomegaly: Positive/Total, 35 Days after Tissue Implant							
Therapy (mg/kg/dose)	Bioassay Time	Spleen (whole)	Thymus (whole)	Marrow (2 femurs)	Nodes (axillary, inguinal)	Blood (ca 1 ml)	Liver (aliquot)	Brain (whole)	Any Tissue
Vincristine, 0.25, days 1 and 5 Prednisone, 30, days 1 and 5 Cyclophosphamide, 100, days 10 and 20 MeCCNU, 16, days 10 and 20	Day 21	3/9	2/10	1/10	0/1	1/9	3/9	3/10	5/10

to our experience with cyclophosphamide plus one of the active nitrosoureas.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Arabinosylcytosine: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytosine arabinoside; ara-C

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Dexamethasone: NSC-34521; CAS reg. No. 50-02-2; pregna-1,4-diene-3,20-dione, 9-fluoro-11β,17,21-trihydroxy-16α-methyl-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

PalmO-ara-C: NSC-135962; CAS reg. No. 31088-06-9; cytosine, 1-β-D-arabinofuranosyl-, 5′-palmitate

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCES

1. FURTH J, SIEBOLD HR, and RATHBONE RR. Experimental studies on lymphomatosis in mice. Am J Cancer 19:521–604, 1933.
2. GROSS L. Oncogenic Viruses, 2nd ed. Oxford, Pergamon Press, 1970, 994 pp.
3. METCALF D, and BRUMBY M. Coulter counter analysis of lymphoma differentiation patterns in AKR mice with lymphoid leukemia. Int J Cancer 2:37–42, 1967.
4. SCHABEL FM, JR. In vivo leukemia cell kill kinetics and "curability" in experimental systems. *In* The Proliferation and Spread of Neoplastic Cells (21st Annual Symposium on Fundamental Cancer Research, 1967, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston). Baltimore, Williams and Wilkins Co., 1968, pp 379–408.
5. VALERIOTE FA, BRUCE WR, and MEEKER BE. Synergistic action of cyclophosphamide and 1,3-bis(2-chloroethyl)-1-nitrosourea on a transplanted murine leukemia. J Natl Cancer Inst 40:935–944, 1968.
6. SKIPPER HE, SCHABEL FM, JR, TRADER MW, ET AL. Response to therapy of spontaneous, first passage, and long passage lines of AK leukemia. Cancer Chemother Rep 53:345–366, 1969.
7. SKIPPER HE, SCHABEL FM, JR, TRADER MW, ET AL. Basic and therapeutic trial results obtained in the spontaneous AK leukemia (lymphoma) model—end of 1971. Cancer Chemother Rep 56:273–314, 1972.
8. GOLDBERG AI, GLYNN JP, KENDE M, ET AL. A two-stage therapeutic design in the spontaneous AKR lymphoma system. Cancer Res 32:1321–1328, 1972.
9. SKIPPER HE, and SCHABEL FM, JR. Spontaneous AK leukemia (lymphoma) as a model for human leukemias and lymphomas. Cancer Chemother Rep Part 3, vol 3:3–5, 1972.
10. LIN H, and BRUCE WR. A cellular approach to the chemotherapy of spontaneous leukemia of AKR mice. Ser Haematol 5:73–88, 1972.
11. FISHER RA, and YATES F. Statistical Tables for Biological, Agricultural, and Medical Research. London, Oliver and Boyd, 1949, 122 pp.
12. KENDE M, GOLDBERG AI, GLYNN JP, ET AL. Relationship between white blood cell count and diagnosis and therapeutic response in AKR mice with spontaneous leukemia (lymphoma). Cancer Chemother Rep 56:683–690, 1972.
13. SCHABEL FM, JR, SKIPPER HE, TRADER MW, ET AL. Spontaneous AK leukemia (lymphoma) as a model system. Cancer Chemother Rep 53:329–344, 1969.
14. SKIPPER HE, SCHABEL FM, JR, and WILCOX WS. Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with "curability" of experimental leukemia. Cancer Chemother Rep 35:1–111, 1964.
15. FREI E III, KARON M, LEVIN RH, ET AL. The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia. Blood 26: 642–656, 1965.
16. HENDERSON ES. Treatment of acute leukemia. Semin Hematol 6:271–319, 1969.
17. SCHABEL FM, JR, SKIPPER HE, LASTER WR, JR, ET AL. Remission induction of spontaneous lymphoma in AKR mice with prednisone + vincristine. Proc Am Assoc Cancer Res 11:69, 1970.
18. GEE TS, YU KP, and CLARKSON BD. Treatment of adult acute leukemia with arabinosylcytosine and thioguanine. Cancer 23:1019–1032, 1969.
19. SCHMIDT LH, MONTGOMERY JA, LASTER WR, JR, ET AL. Combination therapy with arabinosylcytosine and thioguanine. Proc Am Assoc Cancer Res 11:70, 1970.

Combination Radiotherapy and Chemotherapy for P388 Lymphocytic Leukemia In Vivo

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SUMMARY

The antitumor activity of 11 clinically used agents (methotrexate, mechlorethamine, prednisone, alanine mustard, 5-fluorouracil, cyclophosphamide, vinblastine, cytosine arabinoside, vincristine, procarbazine, and *cis*-diamminedichloroplatinum) administered alone and in combination with γ-radiation to mice bearing intraperitoneal or intravenous P388 lymphocytic leukemia demonstrated that combined therapy (with the exception of prednisone) was more effective than γ-radiation or drug therapy alone. The facts also showed that the influence of the timing of drug administration relative to whole-body γ-radiation was not notable except when mechlorethamine was administered just prior to or 4 hours after radiation and when cyclophosphamide was administered 4 hours prior to radiation. The increases in lifespan of tumor-bearing mice obtained with each of the nine other agents were comparable regardless of when the cytotoxic agent was administered in relation to whole-body radiation.

[Cancer Chemother Rep Part 2, vol 4:73–97, 1974]

Many studies in which chemicals or drugs have been combined with radiation in the treatment of tumor-bearing animals or patients have been reported (1–7). It is not our intention in this presentation to summarize the voluminous and exhaustive literature except for a few reports pertinent to the present study.

Chemical agents have been combined with radiation in a variety of ways. Drugs have been administered prior to or at the same time as radiation therapy in an attempt to potentiate the radiation effect (eg, radiosensitizers such as 5-BUdR, 5-IUdR, or BCdR) (8). More recently, Goffinet et al (9) showed a significant increase in the survival time of BCdR-sensitized irradiated animals bearing a mouse glioma compared with controls.

Other antitumor agents administered before or after radiation have been used to potentiate the radiation effect by increasing the cell killing (2,10–20). The rationale for such combinations was that the drug was incorporated into the nucleic acids before or after radiation and would kill neoplastic and normal cells in an additive manner. Perez (21) has reported that marked potentiation and curative effects can be achieved by radiation combined with mechlorethamine, cyclophosphamide, vincristine, and vinblastine in experimental mice with transplanted C3H Gardner lymphosarcoma.[3]

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[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

Unfortunately, these results have not been substantiated in clinical trials, although there have been a few exceptions.

Cytotoxic agents might act by producing a partial synchronization of tumor cells. It is well established in experimental systems that as cells progress through the cell cycle, they exhibit changes in radiosensitivity (22–35). However, this is difficult to produce in the patient, and clinical studies with 5-FU, methotrexate, and hydroxyurea do not appear to be promising. Lampkin (36), however, has reported obtaining a partial synchronization using cytosine arabinoside followed 72 hours later with vincristine. The drugs could inhibit the recovery process which was to take place between the time of radiation and the appearance of injury from a sublethal dose of radiation.

Few studies have thus far been directed at the optimal timing of the drug-radiation interaction although the timing of the drug treatment relative to radiation probably plays a critical role. We have reported that single doses of combinations of hydroxyurea and γ-radiation were additive irrespective of whether radiation was given before or after drug treatment up to 4 hours (37). A greater increase in the survival times of the L1210 tumor-bearing mice was noted with the combined treatment. Valeriote et al (38), using the in vivo spleen assay in AKR mice bearing a transplanted lymphoma, have shown that when 5-FU was administered from 8 hours before to 8 hours after γ-radiation the effect was synergistic.

Andrews (5) has commented that the effectiveness of combined drug and radiation therapy depended not only on the relative radiosensitivity or drug susceptibility of the malignant and normal tissues, but also upon the relative kinetics of these tissues. He also suggested that several types of agents with different biochemical mechanisms of action and different effects with regard to cell-cycle and proliferation kinetics be given in combination with radiotherapy. To answer some of these questions, the preliminary experiments reported in this paper were initiated. They were designed to answer (*a*) whether or not enhancement of survival time of leukemic animals could be obtained by combined radiation and chemotherapy with several types of agents differing in their mechanisms of action, and (*b*) whether or not the time of drug administration relative to γ-radiation was a factor in producing enhancement or toxicity.

MATERIALS AND METHODS

Neoplasm

P388 lymphocytic leukemia originated in an ovariectomized DBA/2 female mouse after painting with methylcholanthrene. Our tumor line was obtained from Microbiological Associates, Inc. (Bethesda, Md), in August 1962, and has been serially transplanted by intraperitoneal (ip) inoculation of 10^6 ascites tumor cells in DBA/2 and CDF_1 hosts. For this study, P388 cells were inoculated ip or intravenously (iv) into CDF_1 (BALB/c female × DBA/2 male) male and female mice at a level of 10^6 cells in a volume of 0.1 ml.

Radiation Source

Groups of leukemic mice were exposed to whole-body γ-radiation by placing them within a perforated circular Lucite cylinder 12″ in diameter and positioning the cylinder in a beam of cobalt 60 γ-radiation from a 3000-curie unit (Tech. Ops. model No. 624). Using lead shields as filters, the whole-body dose was delivered at a mean dose rate of about 150 rads/minute and was uniform to about ±5% over the full size. Dosimetry was checked with a Bendix dosimeter (model No. 686) placed in a phantom mouse.

Drugs

The clinical compounds and dosages employed are shown in table 1. The drugs were administered ip (either as solutions or suspensions in 0.5 ml 24 hours after tumor implantation) in a single dose 4 hours before, just before, or 4 hours after a single dose of whole-body radiation.

RESULTS

Preliminary experiments were carried out to confirm our earlier results with this tumor line. These included the following studies.

TABLE 1.—Compounds and doses tested*

NSC No.	Drugs	Dose (mg/kg/injection)
740	Methotrexate	100, 50, 25, 12.5
762	Mechlorethamine	3.1, 1.56, 0.78, 0.39
10023E	Prednisone	1000, 500, 250, 125
17663	Alanine mustard	12.5, 6.3, 3.1, 1.5
19893	5-FU	200, 100, 50, 25
26271	Cyclophosphamide	400, 200, 100, 50
49842	Vinblastine	12.5, 6.3, 3.15, 1.5
63878	Cytosine arabinoside	1000, 500, 250, 125
67574	Vincristine	3.1, 1.56, 0.78, 0.39
77213	Procarbazine	400, 200, 100, 50
119875	*Cis*-diamminedichloroplatinum	64, 32, 16, 8, 4 4, 2, 1, 0.5

*Drugs were combined with γ-radiation (cobalt 60 source) at doses of 600, 400, 200, and 100 rads.

Cell Inoculum Studies

P388 cells were inoculated at tenfold dilutions to determine the survival time of the tumor-bearing hosts. The results showed an inverse relationship between survival time and dilutions of ip implanted P388 lymphocytic leukemia cells. The doubling time of the exponentially growing tumor cells based on the number of cells inoculated was calculated to be 11.0 hours (fig 1). At a 10^6-cell inoculum, the mean survival time was 11.6 days with the first death occurring on Day 10 and the last death occurring on Day 13. When compared to earlier experiments, these results demonstrated that our tumor system was in good control.

Radiation Studies

The lethality of whole-body γ-radiation was determined in CDF_1 male mice by administering a single dose at levels of 200–1000 rads to groups of 20 mice. The LD50/30 (ie, the dose required to kill 50% of the mice by Day 30) was calculated to be 643.1 ± 0.12 rads with a 95% confidence range of 550–750 rads. At a dose of 600 rads, 40% of the mice died with only one mouse dying on Day 10. Subsequent deaths occurred between Days 17 and 26. Higher doses produced 100% mortality (fig 2). These data showed that it was possible to distinguish between tumor deaths and radiation deaths since mice exposed to total-body γ-rays in the 600–1000-rad dose range die because of destruction of the hematopoietic cell-renewal system which usually occurs in the second or third week after radiation.

Table 2 shows that the P388 cells inoculated ip in CDF_1 mice were sensitive to whole-body γ-radiation at doses of 200–800 rads when γ-rays were administered on various days after tumor implantation. The highest value, a 65% increase in lifespan (ILS), was obtained 24 hours after tumor implant at a dose of 600 rads.

The P388 leukemia remained sensitive to γ-rays when the dose of radiation was administered on subsequent days (Days 2–9 respectively). The optimal dose was 600 rads.

Combined Therapy Studies

The experimental design for the combined therapy studies is shown in figure 3. Briefly, one million cells were inoculated ip or iv 24 hours prior to γ-radiation. Drug at four or five dose levels was administered in a single dose 4 hours before, just before, or 4 hours after γ-radiation. Radiation was given at

TABLE 2.—Survival times of tumor-bearing mice receiving whole-body γ-radiation*

Dose (rads)	γ-Rays administered on Day—								
	1	2	3	4	5	6	7	8	9
0	10.0†	—	—	—	—	—	—	—	—
200	13.0	12.0	12.0	12.0	12.0	11.0	11.0	13.0	11.0
400	14.0	13.0	13.0	12.0	13.0	13.5	12.0	13.5	11.0
600	16.5	14.0	14.5	13.0	13.0	13.0	13.0	13.0	13.0
800	13.0	13.5	13.0	13.5	13.5	12.0	12.0	12.0	15.0
1000	8.5	13.0	12.5	13.0	13.0	11.0	12.0	10.0	14.5

*10^6 P388 cells inoculated ip in 0.1 ml; 20 mice per group.
†Median survival time (days).

doses of 100, 200, 400, and 600 rads. A single experiment consisted of 40 control mice and 24 groups of ten mice each.

The following definitions are given for use in this paper:

Enhancement.—The activity of the combined treatment is greater than that obtained with either agent alone.

Additivity.—The combined therapy treatment equals the addition of the effect of each agent.

Synergism.—The combined effect of the two agents is greater than the addition of the effect of each agent by an increase of greater than 25% or the number of survivors at the end of the experiment is greater.

The antitumor activity of the 11 agents administered alone and in combination with γ-radiation to mice bearing ip P388 lymphocytic leukemia is summarized in table 3.

With the exception of prednisone, combined therapy was more effective than γ-radiation or drug therapy alone. The results also showed that the influence of the timing of drug administration relative to whole-body γ-radiation was not notable except when mechlorethamine

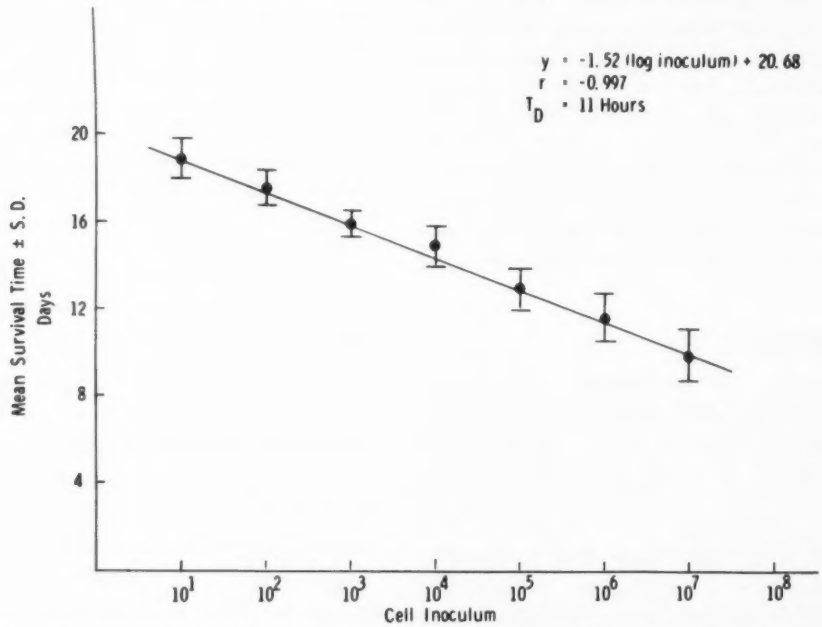

FIGURE 1.—Mean survival times of mice inoculated with various cell numbers of P388 lymphocytic leukemia.

TABLE 3.—Activity of selected agents administered alone and in combination with γ-radiation to mice bearing ip P388 lymphocytic leukemia

NSC No.	Drug	Optimal %ILS (γ-radiation alone)	Optimal %ILS (drug alone)	Optimal %ILS (combination)			Effect
				Drug given 4 hrs before γ-radiation	Drug given just prior to γ-radiation	Drug given 4 hrs after γ-radiation	
740	Methotrexate	45	79	100	108	129	Additive
762	Mechlorethamine	29	66	104	166	183	Synergistic
10023E	Prednisone	41	0	33	33	24	No effect
17663	Alanine mustard	49	95	127	131	113	Enhancement
19893	5-FU	50	55	80	80	90	Enhancement
26271	Cyclophosphamide	22	277	409	322	368	Synergistic
49842	Vinblastine	45	59	91	127	118	Additive
63878	Cytosine arabinoside	23	27	73	82	95	Synergistic
67574	Vincristine	40	63	90	90	113	Additive
77213	Procarbazine	49	9	59	81	59	Additive
119875	*Cis*-diamminedichloroplatinum	50	100	104	114	114	Enhancement

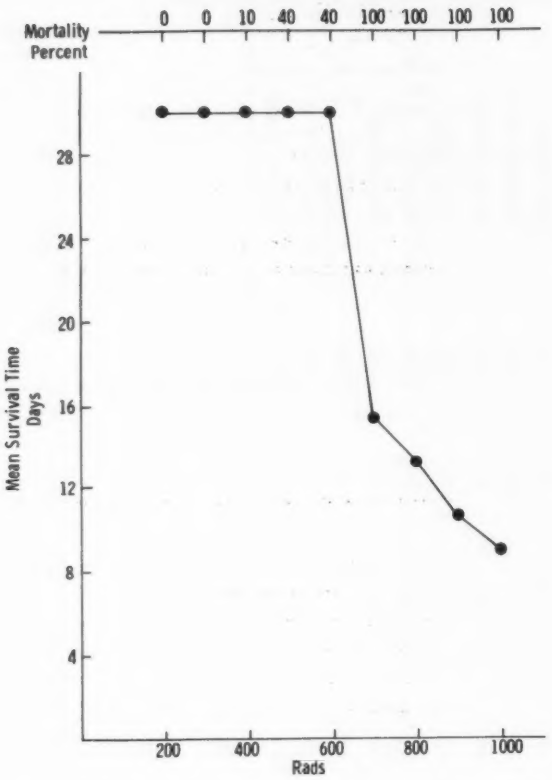

FIGURE 2.—Lethality of whole-body γ-radiation in CDF_1 mice.

was administered just before or 4 hours after radiation and when cyclophosphamide was administered 4 hours before radiation. Comparable ILSs were obtained with each of the nine other agents regardless of when the cytotoxic agent was administered in relation to whole-body radiation.

A similar experiment conducted in mice inoculated iv with P388 cells (table 4) showed that cyclophosphamide was equally effective

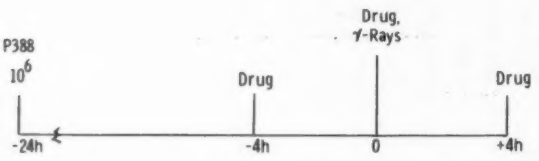

FIGURE 3.—Experimental design for combination radiotherapy and chemotherapy studies.

regardless of the time of drug administration. However, when cyclophosphamide was administered just prior to radiation, there were ten long-term survivors of ten treated animals. Cytosine arabinoside alone was much more effective against the iv inoculated tumor than against the ip inoculated tumor. There was an ILS of 118%. When cytosine arabinoside was administered just before or 4 hours after whole-body γ-radiation, the animals survived much longer (ILS = 231% and 212% respectively). There were no significant differences for each drug in the timing of the administration of the drug relative to radiation in the other combination studies.

It should be remembered that these summary results are essentially single points on the dose response curve and were selected for their maximum ILS. They do not reflect the influence of the timing of drug administration on the complete dose response. To demonstrate the influence of the timing of drug administration, we have selected six of the 11 compounds for detailed review (methotrexate, mechlorethamine, 5-FU, cyclophosphamide, cytosine arabinoside, and vincristine).

Methotrexate and γ-Radiation vs Ip Implanted P388 Leukemia

The results indicated that combined therapy with methotrexate (12.5 mg/kg) and γ-radiation (400 and 600 rads) increased the lifespans of tumor-bearing mice above that seen with methotrexate or radiation alone. However, the timing of the drug administration relative to whole-body γ-radiation (fig 4A) did not influence the chemotherapeutic enhancement.

Combination therapy with methotrexate (25 mg/kg) was, in most cases, less effective than treatment with the drug alone. The timing of the methotrexate treatment had no influence on the combination therapy antitumor effectiveness (fig 4B).

Methotrexate at 50 mg/kg and 400-rad γ-radiation demonstrated an increase in the survival time of tumor-bearing mice higher than that seen with methotrexate or γ-radiation alone when the drug was administered at the same time as or 4 hours after radiation. A

TABLE 4.—Activity of selected agents administered alone and in combination with γ-radiation in mice bearing iv P388 lymphocytic leukemia

NSC No.	Drug	Optimal %ILS (γ-radiation alone)	Optimal %ILS (drug alone)	Optimal %ILS (combination)			Effect
				Drug given 4 hrs before γ-radiation	Drug given just prior to γ-radiation	Drug given 4 hrs after γ-radiation	
740	Methotrexate	33	77	61	88	100	Enhancement
762	Mechlorethamine	37	25	75	62	62	Enhancement
19893	5-FU	37	62	62	62	62	No effect
26271	Cyclophosphamide	37	>650 (6/10*)	>650 (7/10)	>650 (10/10)	>650 (6/10)	Synergistic
49842	Vinblastine	50	50	75	62	93	Enhancement
63878	Cytosine arabinoside	50	118	156	231	212	Synergistic
77213	Procarbazine	37	12	62	56	56	Additive
119875	*Cis*-diamminedichloroplatinum	56	61	105	89	89	Enhancement

*No. of 60-day survivors/No. of mice inoculated.

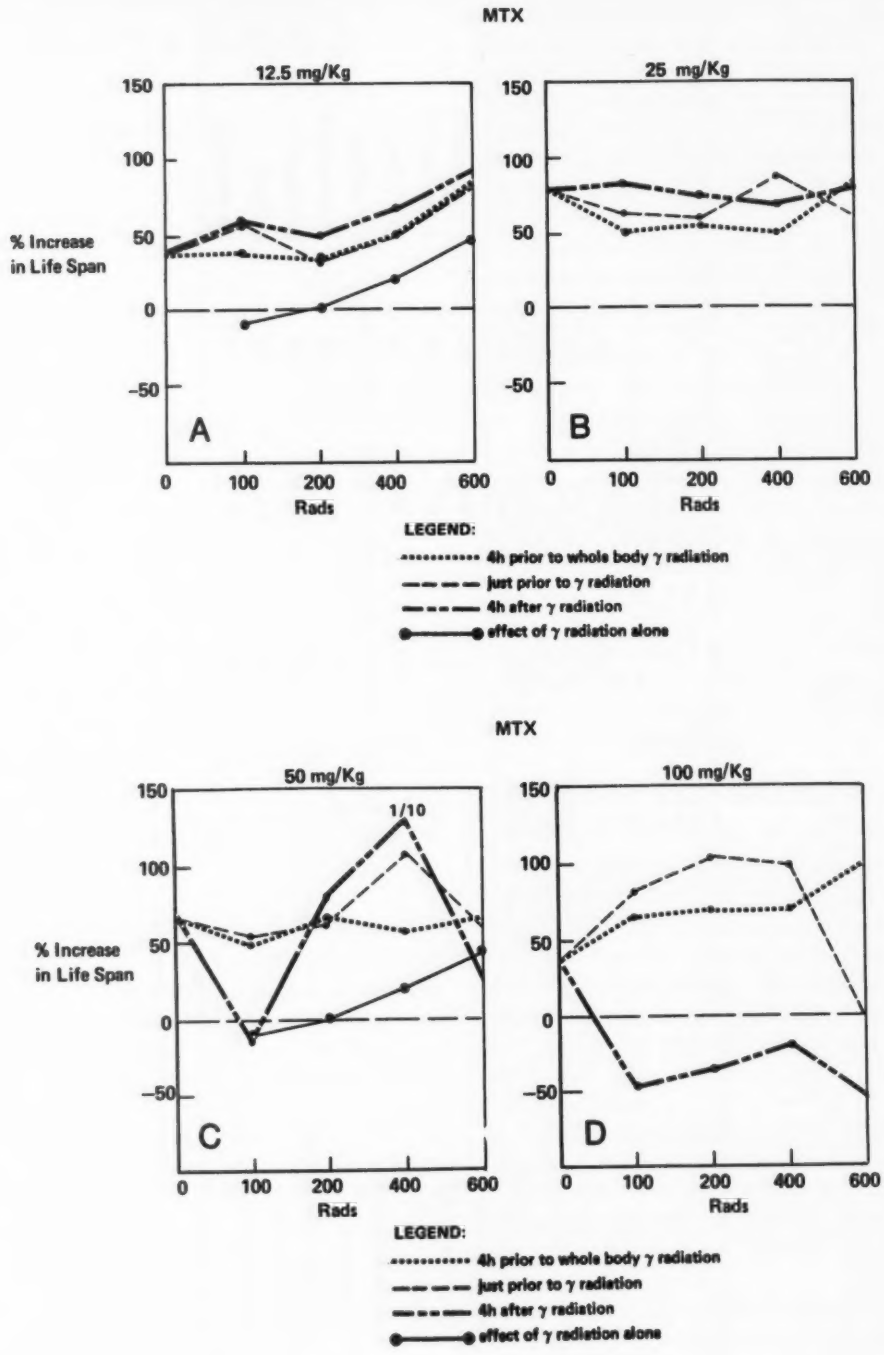

FIGURE 4.—Influence of the timing of administration of methotrexate (MTX) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

higher dose of γ-radiation showed decreased chemotherapeutic effectiveness (fig 4C).

When the tumor-bearing mice received an injection of 100 mg/kg of methotrexate 4 hours prior to γ-radiation, there was an increase in survival time higher than that seen with methotrexate or γ-radiation alone at all combination levels.

Significant increases were obtained with the combined therapy at the 100-, 200-, and 400-rad dosage levels when methotrexate was administered just prior to radiation. A combination of 100 mg/kg of the drug and 600 rads of γ-radiation was toxic for the leukemic mice.

All combinations of 100 mg/kg of methotrexate and γ-radiation produced toxicity when the drug was administered 4 hours after radiation (fig 4D).

Methotrexate and γ-Radiation vs Iv Implanted P388 Leukemia

In general, there were small ILSs in the tumor-bearing mice when the drug was administered at 12.5 or 25 mg/kg at the same time as or 4 hours after γ-radiation. When 50 mg/kg of methotrexate was injected ip 4 hours after radiation, the therapy was more effective than methotrexate or γ-radiation alone at the 200-, 400-, and 600-rad levels. All therapies were toxic at 100 mg/kg (fig 5[A–D]).

Mechlorethamine and γ-Radiation vs Ip Implanted P388 Leukemia

Mechlorethamine (0.39 mg/kg) produced slight increases in survival times when combined with 400 and 600 rads of γ-radiation regardless of when the drug was administered (fig 6A). At 0.78 mg/kg, the maximal effect, an 87% ILS over controls, was obtained when the drug was administered just prior to 100 rads of γ-radiation (fig 6B). Combined therapy with 1.56 mg/kg of mechlorethamine and 400 rads of γ-radiation was optimal when the drug was also administered just prior to whole-body radiation (fig 6C).

Increased survival times were also obtained with the 3.1-mg/kg mechlorethamine combination therapy regardless of the timing of drug administration. The optimal ILS occurred when mechlorethamine was administered 4 hours after 400 rads of γ-radiation. There was a 183% ILS in tumor-bearing mice over the controls with two survivors of ten treated animals (fig 6D). However, this was not significantly different from the combined therapy with 100 rads when mechlorethamine was administered just prior to γ-radiation.

Figure 7 shows that the combination of mechlorethamine at 3.1 mg/kg and γ-radiation at 400 rads was much more effective when mechlorethamine was administered at the same time as or 4 hours after γ-radiation. Administration of the drug prior to radiation increased the toxicity for the tumor-bearing mice.

Fifty percent of the mice died by Day 19 when 3.1 mg/kg of mechlorethamine was given alone. The combined therapy treatments showed that mechlorethamine given 4 hours prior to γ-rays was toxic, 50% of the animals dying by Day 5. When mechlorethamine was given just prior to or 4 hours after radiation, the 50% endpoints were 25 and 32 days respectively. In addition, there were three of ten and one of ten long-term survivors.

Mechlorethamine vs Iv Implanted P388 Leukemia

Mechlorethamine was less effective in mice implanted iv with P388 cells. In the experiments using 0.39 mg/kg of mechlorethamine plus γ-radiation, only the 400- and 600-rad doses were more effective than γ-radiation alone or drug alone (fig 8A). ILSs similar to those seen with radiation alone were obtained with higher doses of mechlorethamine and γ-radiation (fig 8[B,C,D]).

5-FU vs Ip Implanted P388 Leukemia

The time of 5-FU treatment did not influence the potentiation of the combination treatment. Longer ILSs with the combined therapy were obtained with 25 or 50 mg/kg of 5-FU and 400 or 600 rads of γ-radiation. When the dose of 5-FU was increased to 100 mg/kg, enhancement was observed only in combination with 200 rads of γ-radiation. Higher doses of radiation-drug combinations showed a decrease in survival time. Toxicity was noted with all schedules using 200 mg/kg of 5-FU with one exception: when this dose was administered 4 hours prior to γ-radiation (100

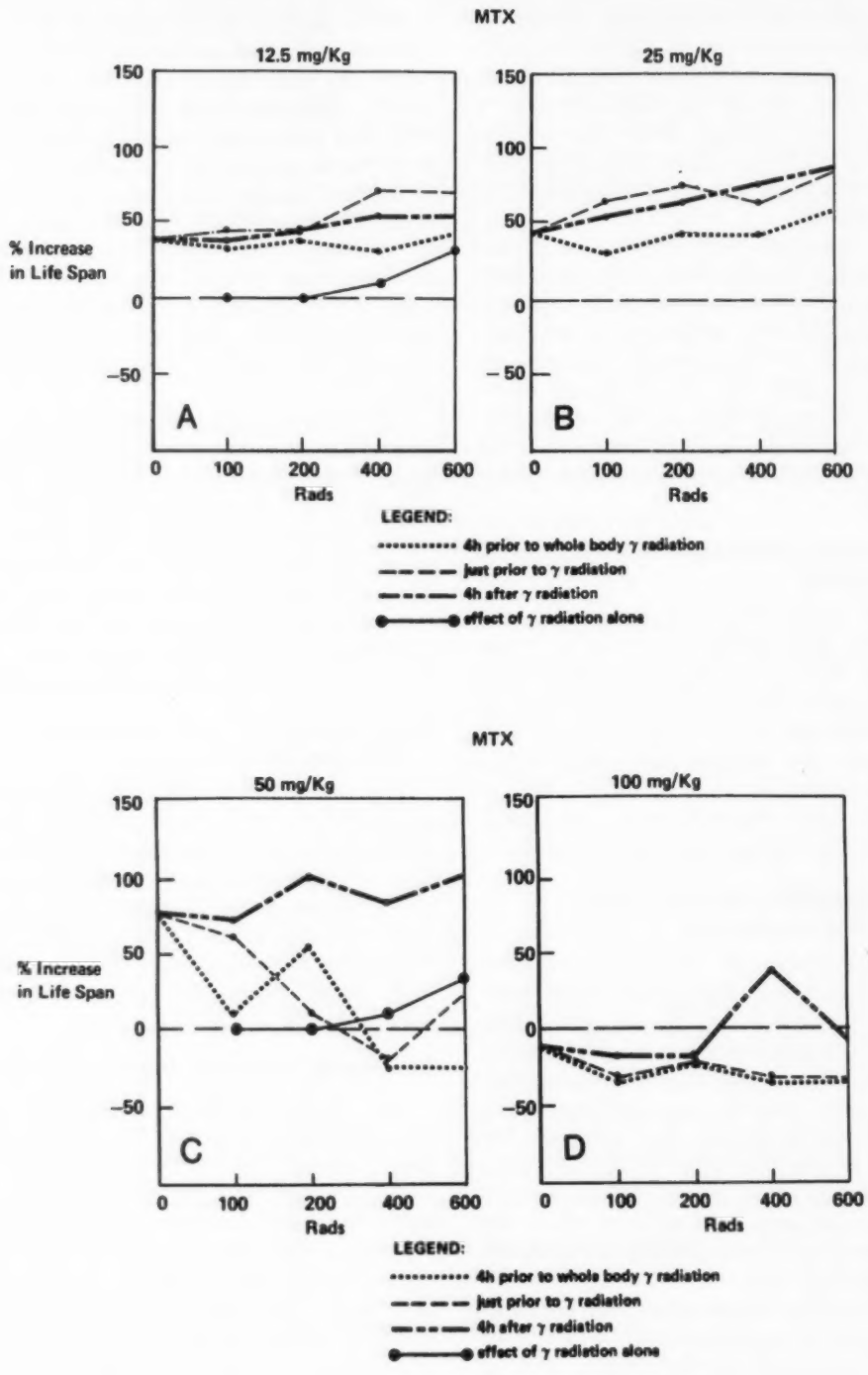

FIGURE 5.—Influence of the timing of administration of methotrexate (MTX) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

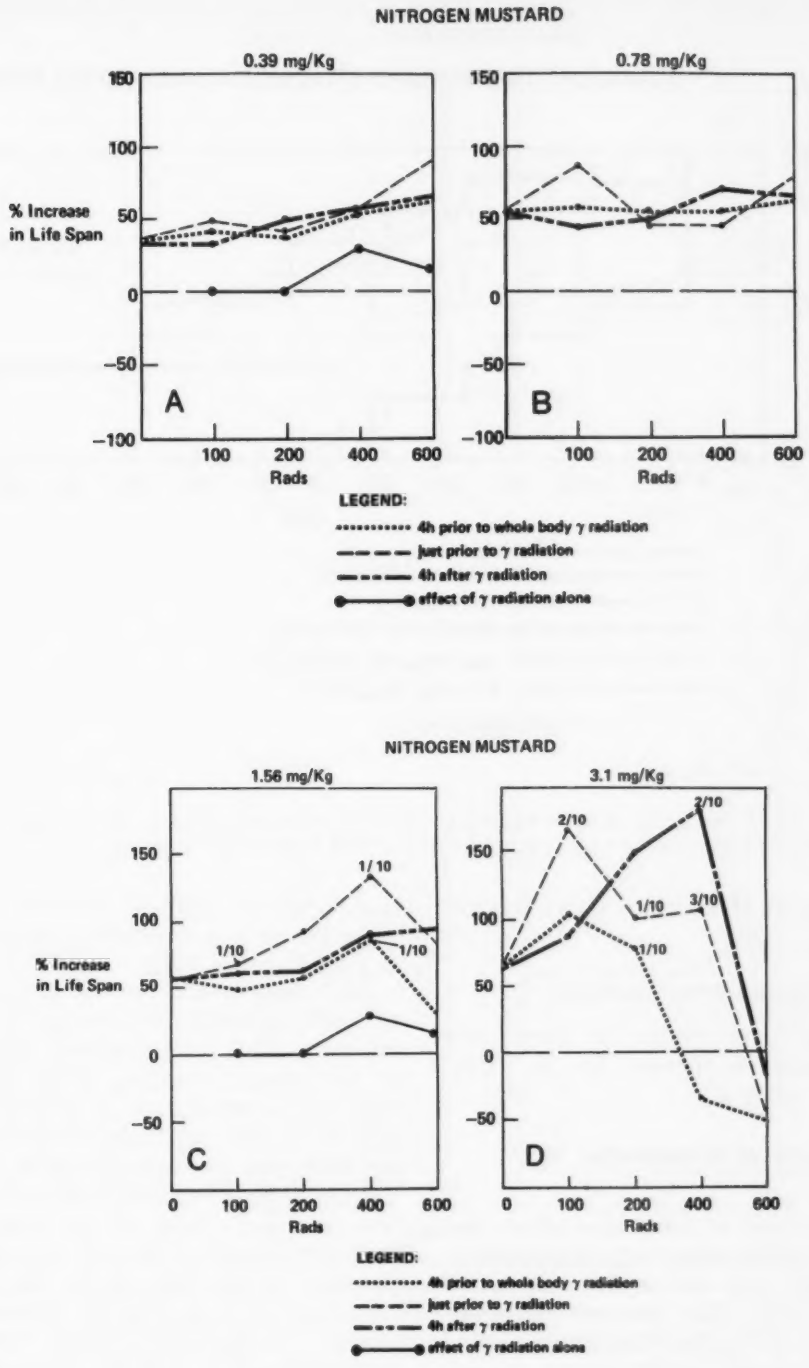

FIGURE 6.—Influence of the timing of administration of mechlorethamine (nitrogen mustard) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

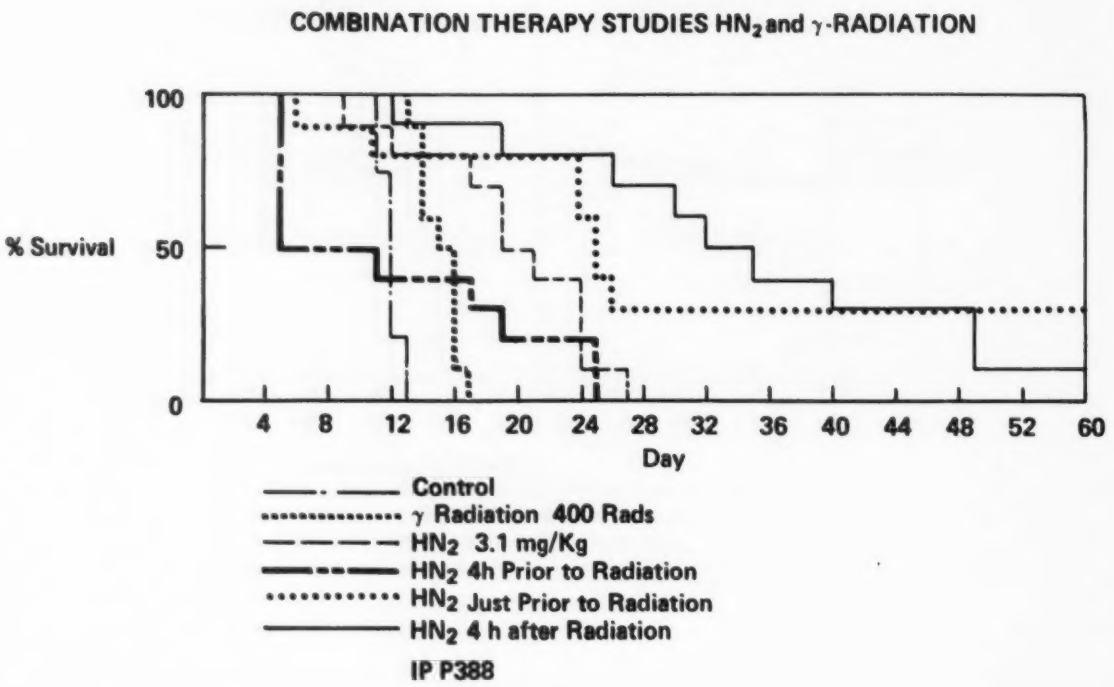

FIGURE 7.—Influence of the timing of administration of mechlorethamine (HN_2) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

rads), there was an ILS of 45% above controls (fig 9[A–D].

5-FU vs Iv Implanted P388 Leukemia

These results were similar to those seen with the combination therapy for ip inoculated P388 (fig 10[A–D]).

Cyclophosphamide vs Ip Implanted P388 Leukemia

Slight enhancement of antitumor effect was obtained with combinations of cyclophosphamide (50 mg/kg) and 400 or 600 rads of γ-radiation (fig 11A). The time of treatment was not a factor. When the dose of cyclophosphamide was increased to 100 mg/kg, longer survival times were noted with the 100- and 200-rad γ-radiation combinations. In addition, a number of the combinations produced 60-day survivors. Optimal therapy was obtained when the drug was administered 4 hours prior to radiation (fig 11B).

At a dose of 200 mg/kg, cyclophosphamide by itself increased the lifespan of tumor-bearing mice 277% and produced three survivors of ten treated animals. When cyclophosphamide was administered 4 hours prior to 200 rads of γ-radiation, there were five of ten 60-day survivors. Cyclophosphamide administered 4 hours after 100-, 200-, and 400-rad γ-radiation produced three of ten survivors. The ILSs were longer at the 100- and 400-rad combination therapy dose levels (fig 11C). However, there was no clearcut evidence that the timing of drug administration influenced the chemotherapeutic activity. Cyclophosphamide (400 mg/kg) in combination with 200, 400, and 600 rads shortened the lifespans of the tumor-bearing mice (fig 11D).

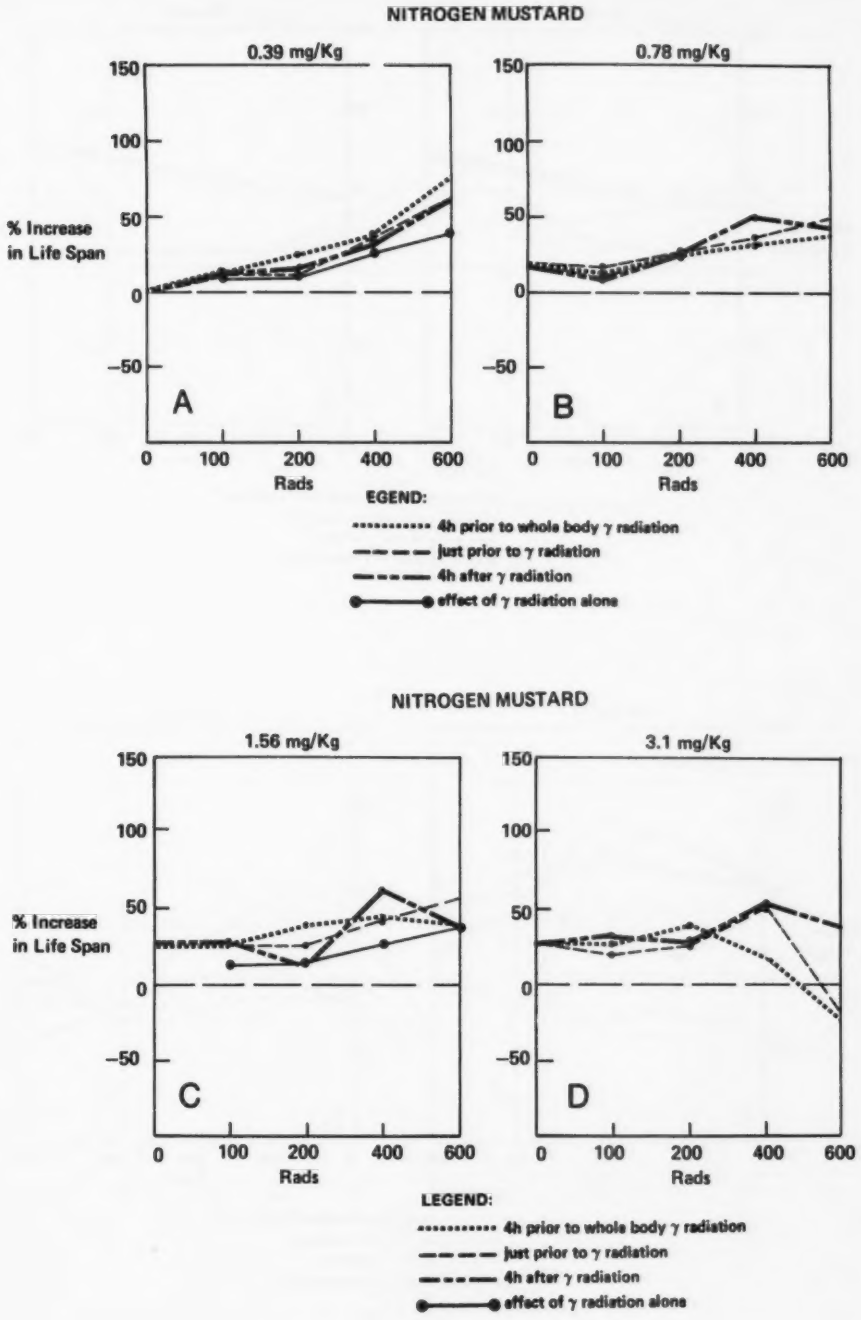

FIGURE 8.—Influence of the timing of administration of mechlorethamine (nitrogen mustard) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

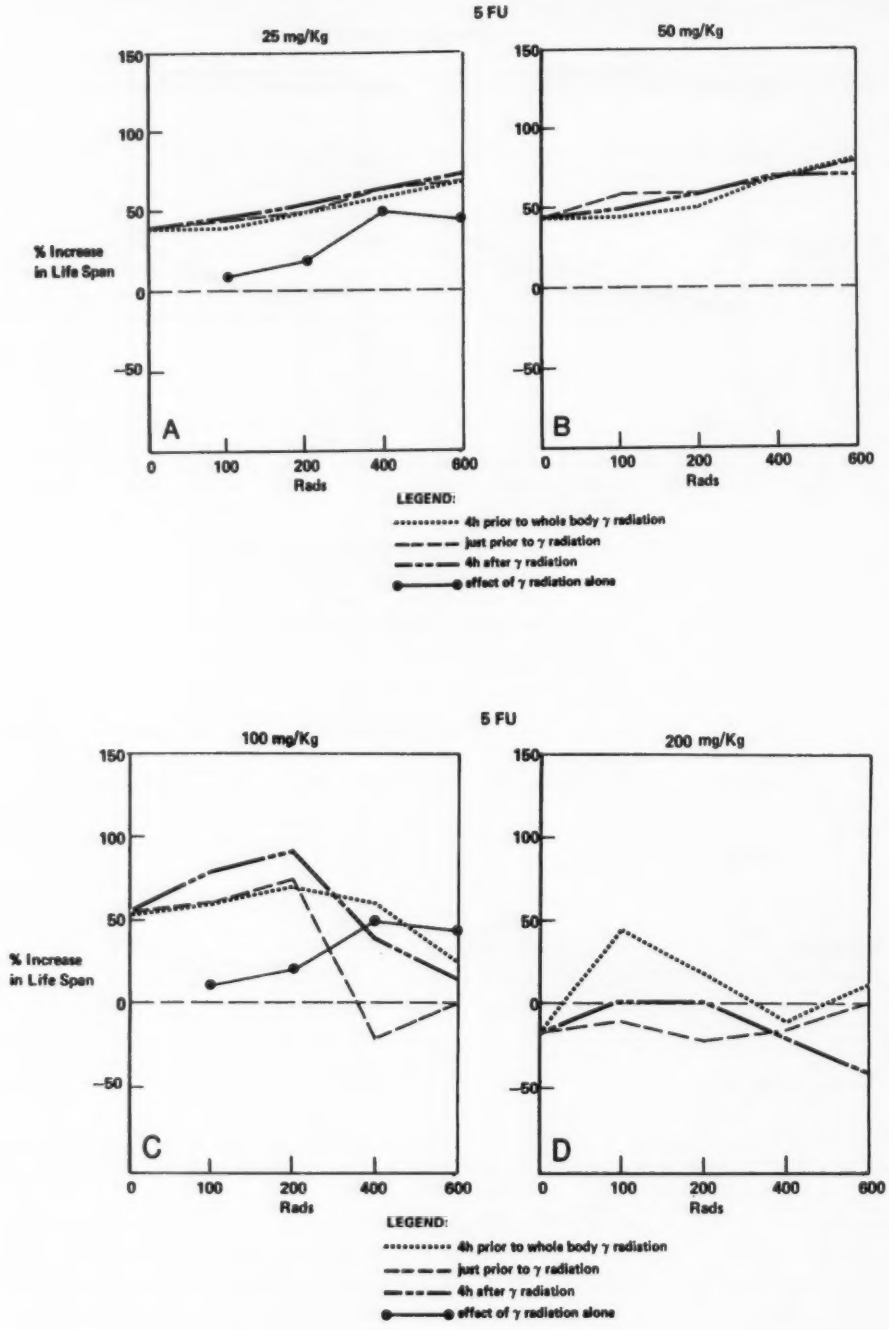

FIGURE 9.—Influence of the timing of administration of 5-FU on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

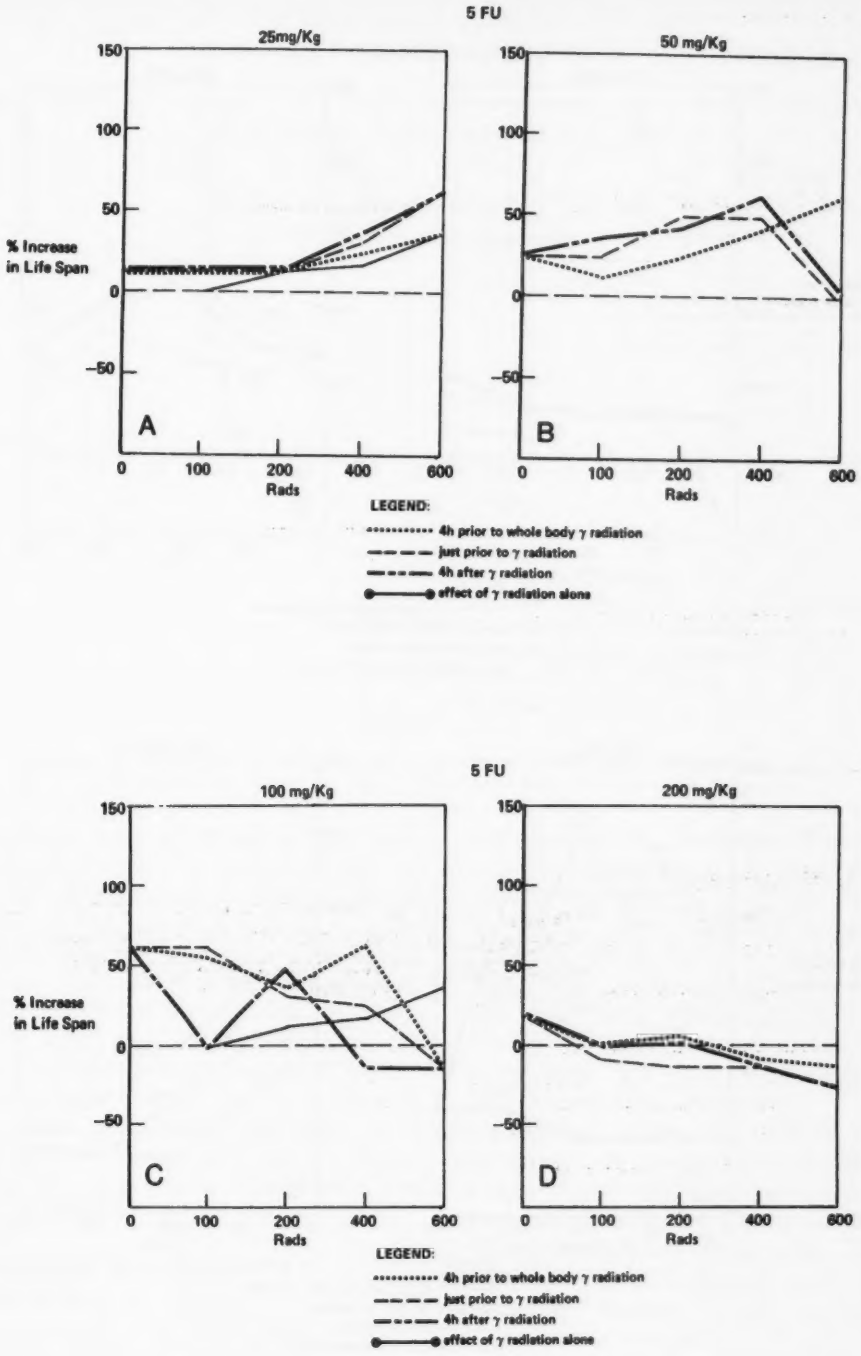

FIGURE 10.—Influence of the timing of administration of 5-FU on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

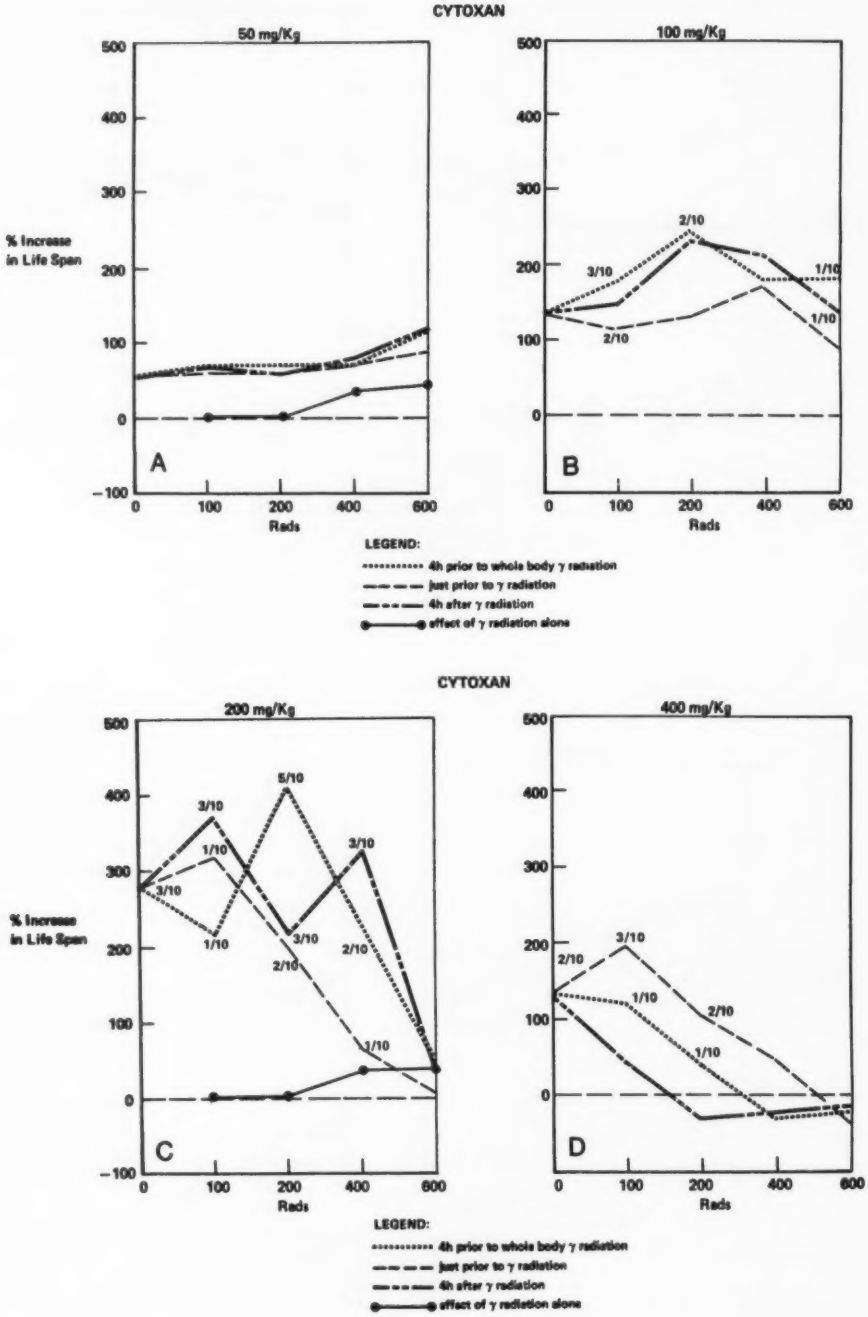

FIGURE 11.—Influence of the timing of administration of cyclophosphamide (Cytoxan) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

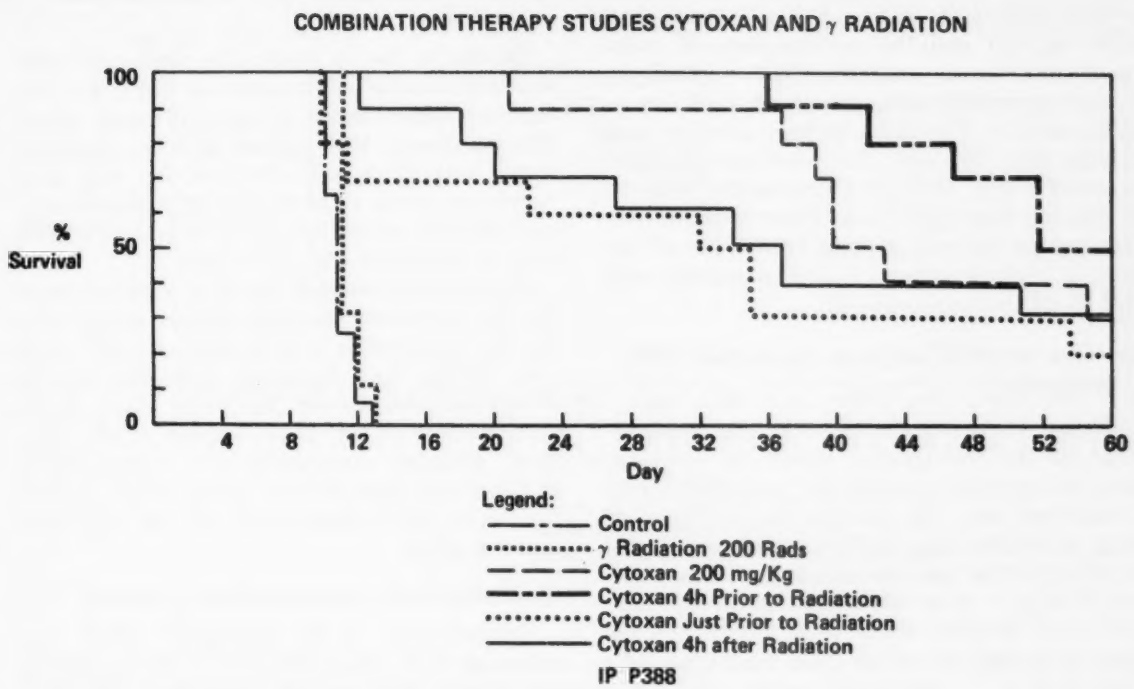

FIGURE 12.—Influence of the timing of administration of cyclophosphamide (Cytoxan) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

The individual days of death of the mice treated with 200 mg/kg of cyclophosphamide and 200 rads of radiation were also plotted (fig 12). The results showed that there were no differences between the control mice and those groups that received radiation alone. The 50% survival point was 43 days for cyclophosphamide alone and 32 and 34 days for the combination therapy in those groups that received cyclophosphamide at the same time as or 4 hours after γ-radiation. However, when cyclophosphamide was administered 4 hours prior to radiation, the 50% survival point was reached at 52 days.

Cyclophosphamide vs Iv Implanted P388 Leukemia

There was a 268% ILS of animals above that seen with cyclophosphamide alone (fig 13A) when 50 mg/kg was administered 4 hours prior to 600-rad γ-radiation. Enhancement was also noted with 50 mg/kg of cyclophosphamide and 400 rads of γ-radiation. The therapy combinations using 100 mg/kg and 400 rads were more effective, with seven of ten and six of ten 60-day survivors when cyclophosphamide was administered 4 hours prior to or at the same time as γ-radiation. In combination with 600 rads, cyclophosphamide was curative for all the mice; ten of ten animals survived when the drug was administered just prior to radiation (fig 13B).

Figure 14 shows the days of death of the individual mice receiving combined therapy at a dose of 100 mg/kg of cyclophosphamide and 400 rads of radiation. There was no significant difference between those mice that received cyclophosphamide alone or were treated with combined therapy when cyclophosphamide was given 4 hours after radiation. The optimal schedules for this combination occurred when cyclophosphamide was administered 4 hours prior to or at the same time as radiation.

Figure 15 shows that there was no signifi-

cant difference in antitumor effect between cyclophosphamide alone at the optimal dose (200 mg/kg) and the combinations of cyclophosphamide and γ-radiation (ILS = >650%). Cyclophosphamide alone produced a 40% mortality by Day 33 with a median survival time greater than 60 days. The combined therapies produced only 10%–20% mortality with 26 of 30 long-term survivors. There were no differences in the end results regardless of the time of cyclophosphamide administration relative to whole-body radiation.

Cytosine Arabinoside vs Ip Implanted P388 Leukemia

Figure 16 demonstrates that the enhancement of the therapeutic effects by combinations of cytosine arabinoside and whole-body γ-radiation was not related to the time of drug administration. Enhancement was noted on all schedules and the maximal ILS of 95% was obtained with the combination of 1000 mg/kg of cytosine arabinoside given 4 hours after 600 rads of whole-body radiation (fig 16[A–D]).

Cytosine Arabinoside vs Iv Implanted P388 Leukemia

Similar to the ip study, the timing of cytosine arabinoside administration did not influence the enhancement of the antitumor effect. The maximum ILS against the iv implanted tumor was 231% with three of ten long-term survivors when 1000 mg/kg of cytosine arabinoside was given just prior to a dose of 600 rads of radiation (fig 17[A–D]).

Figure 18 shows that the 50% survival point for the combined therapy studies using 1000 mg/kg of cytosine arabinoside and 600 rads was similar and probably not significantly different from that of the drug alone. However, the subsequent days of death of the mice when cytosine arabinoside was administered at the same time as or 4 hours after γ-radiation show the enhancement of the combined therapy effect.

Vincristine vs Ip Implanted P388 Leukemia

Enhancement of the antitumor effect was obtained with all combinations of vincristine and 400 or 600 rads of γ-radiation. The ILS

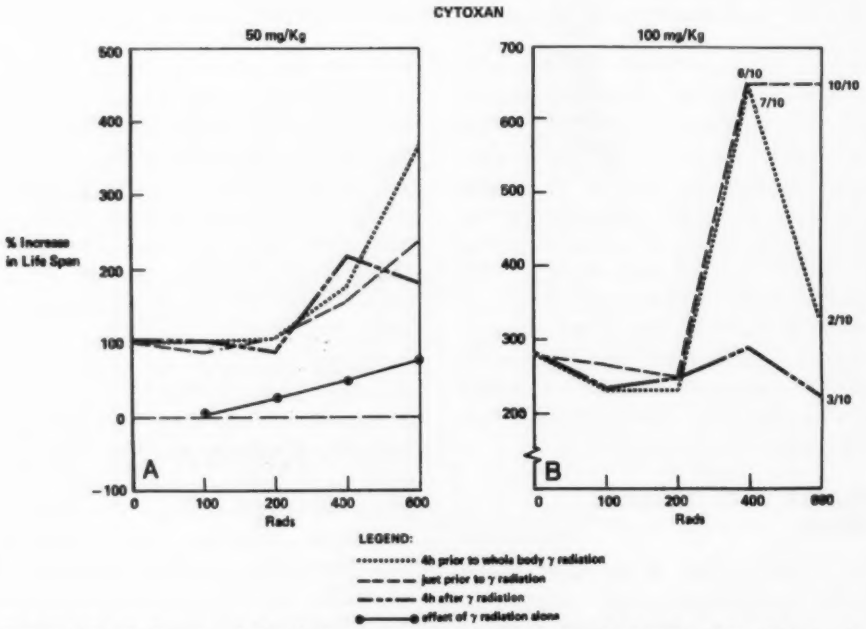

FIGURE 13.—Influence of the timing of administration of cyclophosphamide (Cytoxan) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

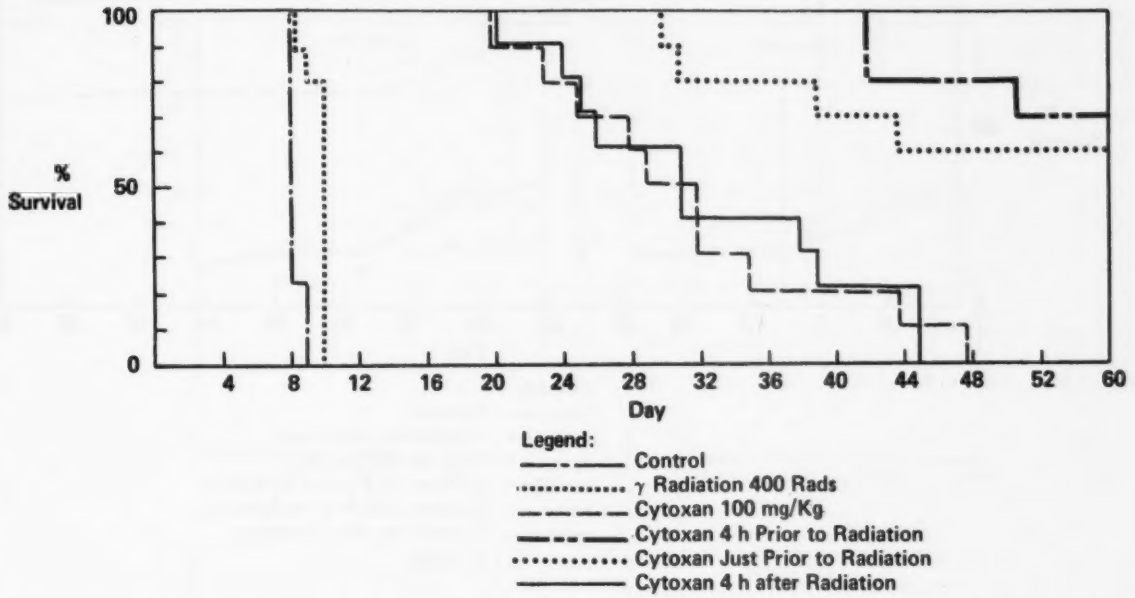

FIGURE 14.—Influence of the timing of administration of cyclophosphamide (Cytoxan) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

of tumor-bearing mice was greater than that obtained with vincristine or γ-radiation alone. One combination, vincristine at 3.1 mg/kg administered just prior to 600 rads of radiation, was toxic for the animals. The timing of the drug administration did not influence the enhanced therapy (fig 19[A–D]).

CONCLUSIONS

In these experiments, we have demonstrated that combined radiotherapy and chemotherapy resulted in increased survival times in mice compared to those observed for drug or γ-radiation alone.

Three drugs, mechlorethamine, cyclophosphamide, and cytosine arabinoside, combined with γ-rays were synergistic in their activity in mice bearing ip implanted P388, and combined therapy with cyclophosphamide and cytosine arabinoside also showed synergism against the iv implanted P388. All other drugs, with the exception of prednisone, produced some degree of enhancement when combined with γ-rays.

The timing of drug administration or the reversing of the sequence of drug and radiation therapy under the conditions reported herein did not significantly alter the ILSs of the tumor-bearing mice for most of the agents tested.

Doses of whole-body γ-radiation close to the maximum tolerated level were necessary when used in combination with chemotherapeutic agents, and increased toxicity was observed for the combined treatment with 600 rads of γ-radiation and higher dose levels of drug. Combined therapy with lower doses of radiation usually indicated no improvement over that seen with drug alone.

Attention will be given in future studies to the use of newer active chemotherapeutic agents, routes and schedules of administration, time relationships between the administration of the drugs and radiation, and the utilization of other tumor systems such as the solid B16 melanoma.

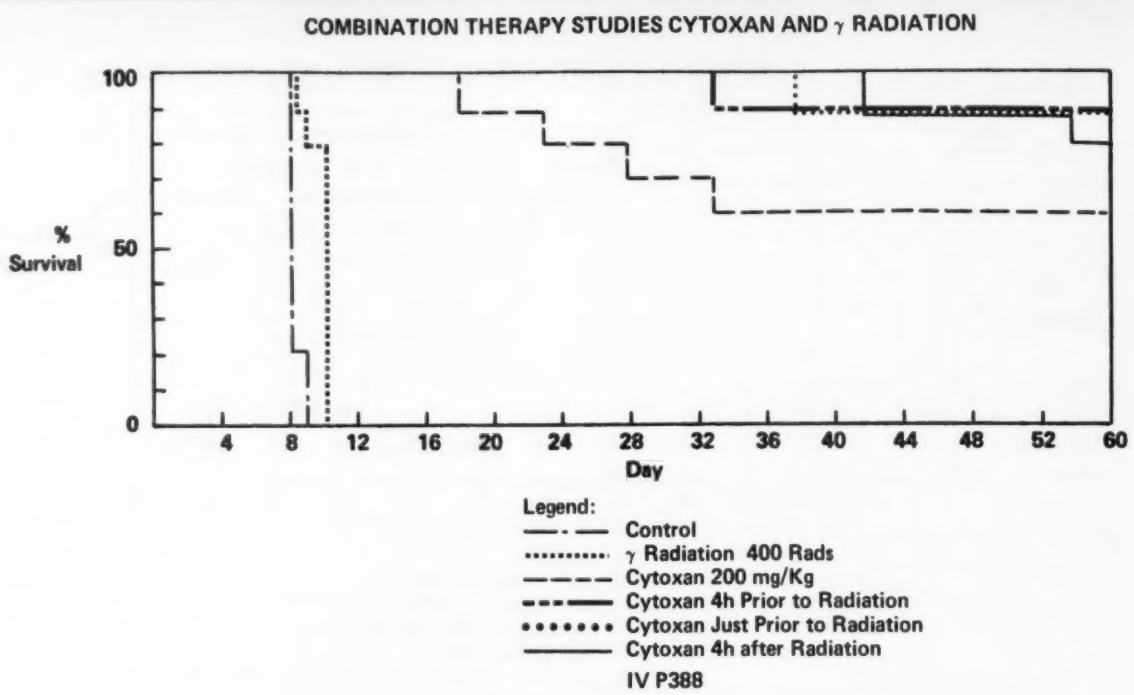

FIGURE 15.—Influence of the timing of administration of cyclophosphamide (Cytoxan) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

LIST OF COMPOUNDS

Alanine mustard: NSC-17663; alanine, *N,N*-bis(2-chloroethyl)-, hydrochloride, DL-

***Cis*-diamminedichloroplatinum:** NSC-119875; CAS reg. No. 15663-27-1

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCES

1. BANE HN, CONRAD JT, and TARNOWSKI GS. Combination therapy of malignant tumors with ionizing radiations and chemicals: a review. Cancer Res 17:551–566, 1957.
2. TARNOWSKI GS, BANE HN, CONRAD J, ET AL. Effects of combinations of radiation and chemotherapeutic agents against experimental animal tumors. Cancer Res 18:225–245, 1958.
3. KLIGERMAN MM. The role of combination radiation and chemotherapy. *In* Proceedings of the Conference on Research on the Radiotherapy of Cancer, University of Wisconsin, 1960. New York, American Cancer Society, 1961, pp 147–157.
4. HODNETT EM. Drugs used with radiation in the treatment of cancer. Cancer Chemother Rep 32:55–65, 1963.
5. ANDREWS JR. Commentary: Combined cancer radiotherapy and chemotherapy: the relevance of cell population kinetics and pharmacodynamics. Cancer Chemother Rep 53:313–324, 1969.
6. STUTZMAN L. Combined radiotherapy and chemotherapy of lymphomas and other cancers. Cancer Res 31:1845–1850, 1971.
7. BLEEHEN NM. Combination therapy with drugs and radiation. Br Med J 29:54–58, 1973.
8. BAGSHAW MA, and DOGGETT RLS. A clinical study

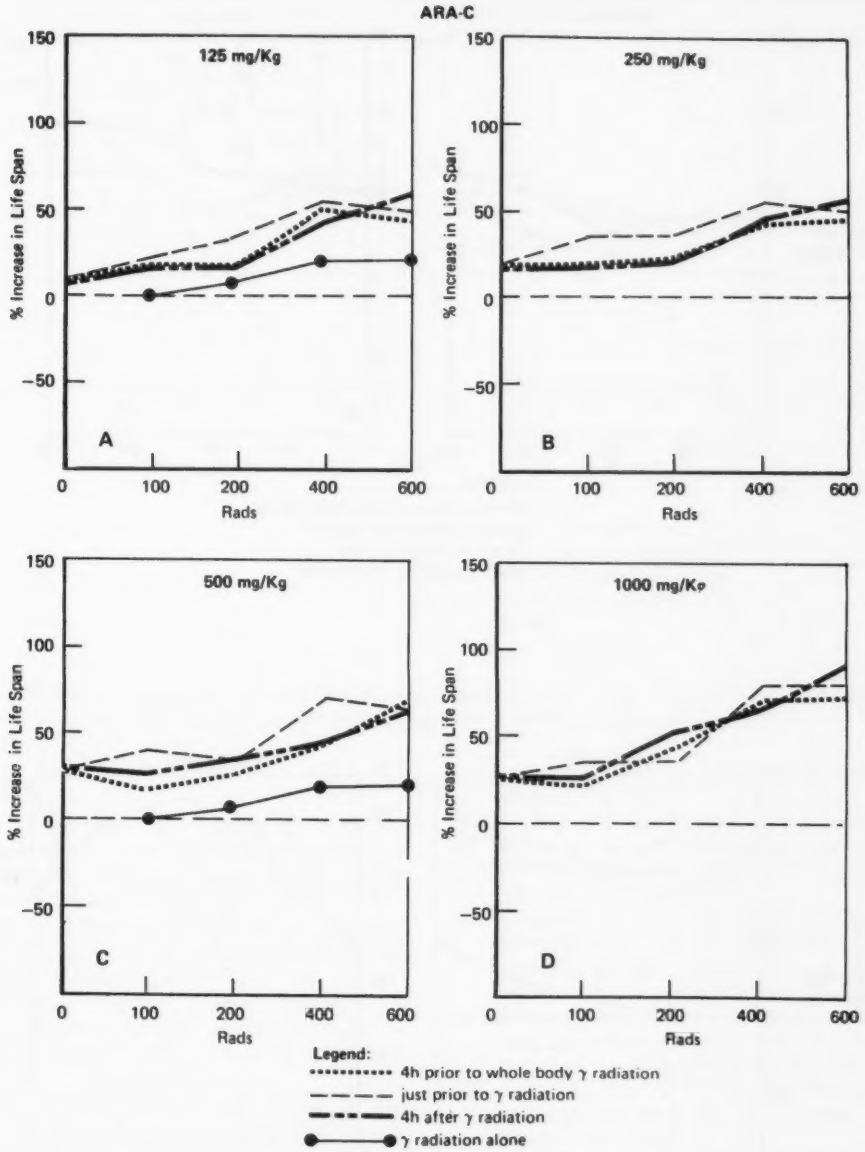

FIGURE 16.—Influence of timing of administration of cytosine arabinoside (ara-C) on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

of chemical radiosensitization. Front Radiat Ther Oncol 4:164–173, 1969.

9. GOFFINET DR, BROWN JM, BAGSHAW MA, ET AL. Prolonged carotid arterial radiosensitizer infusion and radiation therapy of mouse gliomas. Am J Roentgenol Radium Ther Nucl Med 114:7–15, 1972.
10. JOHNSON RE. An experimental therapeutic approach to L1210 leukemia in mice: combined chemotherapy and central nervous system irradiation. J Natl Cancer Inst 32:1333–1341, 1964.
11. DIXON GJ, BLACKWELL MM, and SCHABEL FM, JR. Hadacidin potentiation of lethal action of ionizing

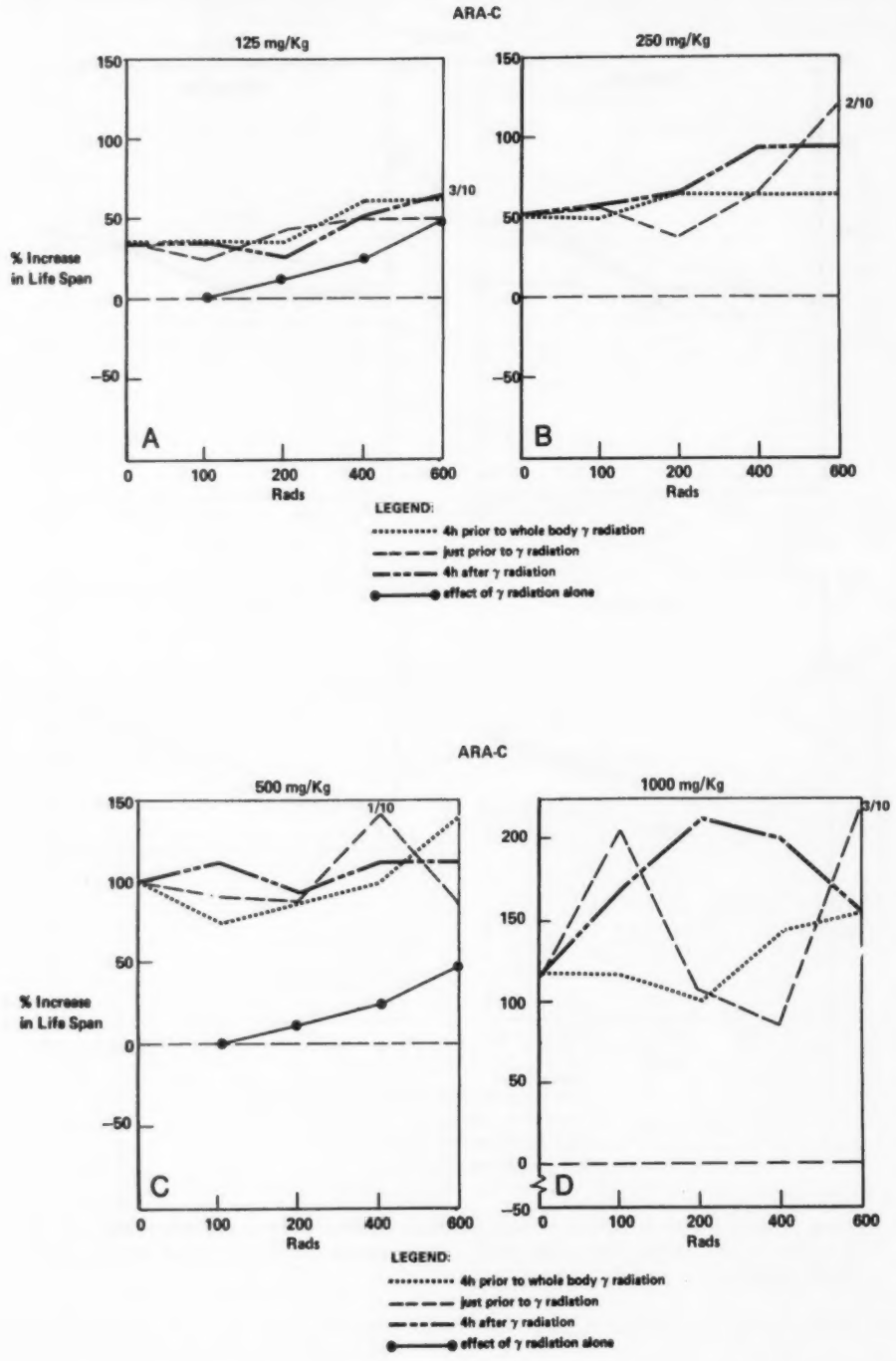

FIGURE 17.—Influence of the timing of administration of cytosine arabinoside (ara-C) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

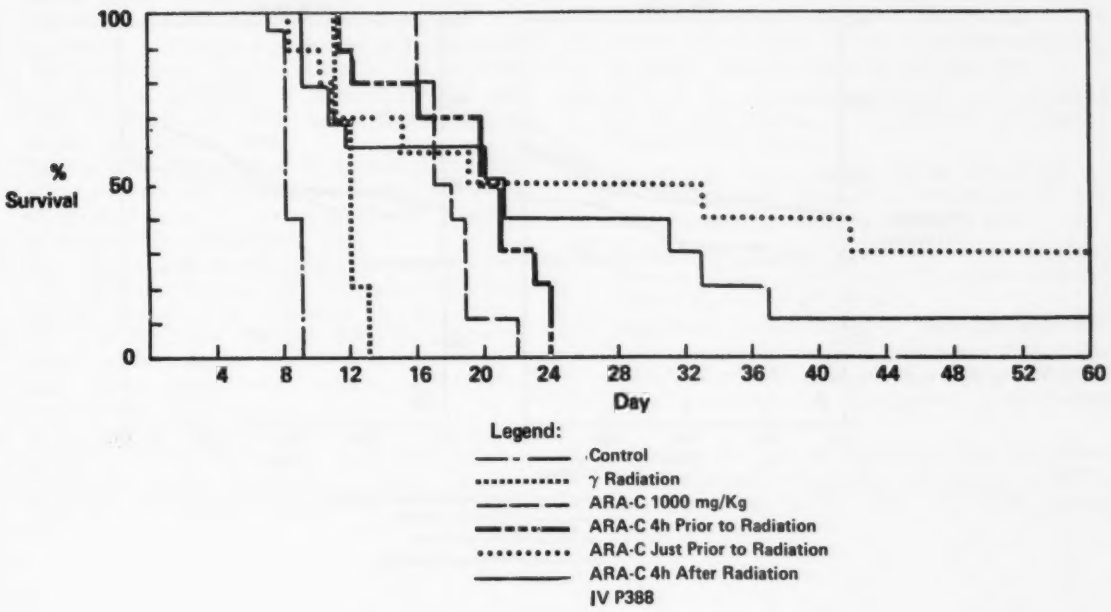

FIGURE 18.—Influence of the timing of administration of cytosine arabinoside (ara-C) on the potentiation of the antitumor effect of γ-radiation in mice bearing iv inoculated P388 lymphocytic leukemia.

irradiation. Proc Soc Exp Biol Med 118:521–525, 1965.

12. D'ANGIO GJ, MADDOCK CL, FARBER S, ET AL. The enhanced response of the Ridgway osteogenic sarcoma to roentgen radiation combined with actinomycin D. Cancer Res 25:1002–1007, 1965.
13. LOKEN MK, KIM YS, MOSSER DG, ET AL. The effect of combined irradiation and chemotherapy on cancer growth. Radiology 73:166–174, 1969.
14. SHOHAT B, GITTER S, LEVIE B, ET AL. The combined effect of cucurbitacins and x-ray treatment on transplanted tumors in mice. Cancer Res 25:1828–1835, 1965.
15. LOTTSFELDT FL, SCHWARTZ S, and KRIVIT W. Hyperbaric oxygen, whole-body x-irradiation and cyclophosphamide combination therapy in mouse leukemia L1210. J Natl Cancer Inst 36:37–43, 1966.
16. HOST H. Comparative effects of cyclophosphamide, nitrogen mustard, and total-body irradiation on survival and on white blood cells in rats. Radiat Res 27:638–651, 1966.
17. VALERIOTE AF, COLLINS DC, and BRUCE WR. Hematological recovery in the mouse following single doses of gamma radiation and cyclophosphamide. Radiat Res 33:501–511, 1968.
18. DONALDSON SS, COOPER RA, JR, and FLETCHER WS. Effect of Coley's toxins and irradiation on the A. melanoma #3 tumor in the golden hamster. Cancer 21:805–811, 1968.
19. ELKIND MM, and SAKAMOTO K. Combined effects of x-irradiation and chemotherapeutic drugs (nitrogen mustard and actinomycin D). Front Radiat Ther Oncol 4:53–75, 1969.
20. KAUFMAN H, and HARDER S. The action of combined treatment with ionising rays and cyclophosphamide on the mitotic rate of Ehrlich ascites tumors. Strahlentherapie 142:677–680, 1971.
21. PEREZ CA, and OLSON J. Preoperative irradiation and chemotherapy in the cure of a mouse lymphosarcoma. Radiology 92: 136–142, 1969.
22. PATT HV, and QUASTLER H. Radiation effects on cell renewal and related systems. Physiol Rev 43:357–396, 1963.
23. WHITMORE GF, and GULYAS S. Studies on recovery processes in mouse L cells. *In* Conference on Radiobiology and Radiotherapy. Natl Cancer Inst Monogr 24:141–156, 1967.
24. POWERS WE, PALMER LA, and TOLMACH LJ. Cellular radiosensitivity and tumor curability. *In* Conference on Radiobiology and Radiotherapy. Natl Cancer Inst Monogr 24:169–185, 1967.
25. KALLMAN RF. Evidence for cyclic fluctuations in radiosensitivity and their implications. *In* Conference on Radiobiology and Radiotherapy. Natl Cancer Inst Monogr 24:205–223, 1967.

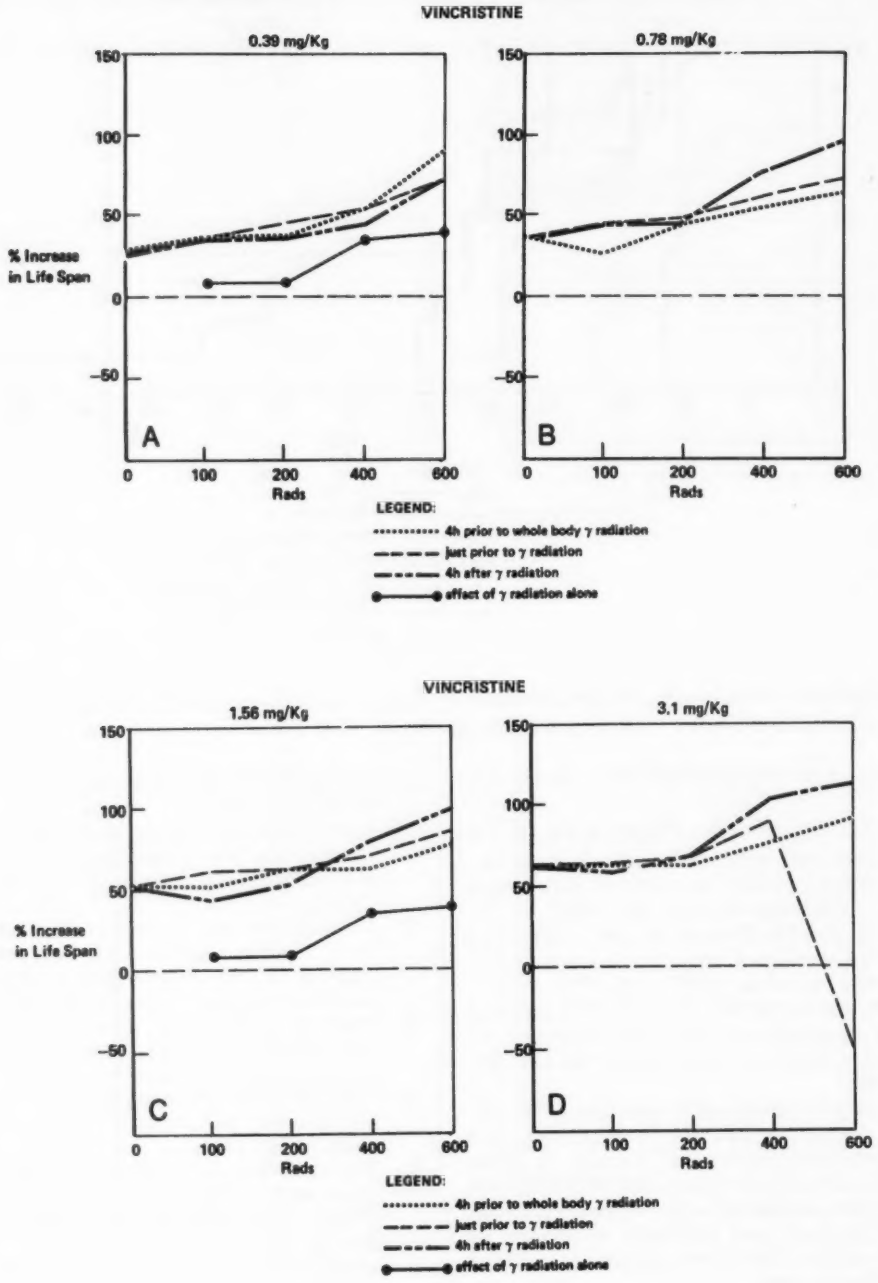

FIGURE 19.—Influence of the timing of administration of vincristine on the potentiation of the antitumor effect of γ-radiation in mice bearing ip inoculated P388 lymphocytic leukemia.

26. DEWEY WC, and HUMPHREY RM. Relative radiosensitivity of different phases in the life cycle of L–P59 mouse fibroblasts and ascites tumor cells. Radiat Res 16:503–530, 1962.
27. GOLDFEDER A. Biological properties and radiosensitivity of tumors: Determination of the cell-cycle and time of synthesis of deoxyribonucleic acid using tritiated thymidine and autoradiography. Nature 207:612–614, 1965.
28. ELKIND MM, and SUTTON H. Radiation response of mammalian cells grown in culture. 1. Repair of x-ray damage in surviving Chinese hamster cells. Radiat Res 13:556–593, 1960.
29. TERASIMA T, and TOLMACH LJ. Variations in several responses of HeLa cells to X-irradiation during the division cycle. Biophys J 3:11–33, 1963.
30. SINCLAIR WK, and MORTON RA. Variations in X-ray response during the division cycle of partially synchronized Chinese hamster cells in culture. Nature 199:1158–1160, 1963.
31. WHITMORE GJ, GULYAS S, and BOTOND J. Radiation sensitivity throughout the cell cycle and its relationship to recovery. *In* Cellular Radiation Biology. Baltimore, Williams & Wilkins, Co., 1965, pp 423–441.
32. DEWEY WC, and HUMPHREY RM. Radiosensitivity and recovery of radiation damage in relation to the cell cycle. *In* Cellular Radiation Biology. Baltimore, Williams & Wilkins, Co., 1965, pp 340–375.
33. LITTLE JB. Cellular effects of ionizing radiation. N Engl J Med 278:308–315, 369–376, 1968.
34. BRYANT PE. Changes in sensitivity of cells during exposure to radiation at low dose-rate. Int J Radiat Biol 22:67–73, 1972.
35. TROTT K-R. Some radio-biological aspects to the "Synchronization Therapy in Treatment of Tumors." Strahlentherapie 142:568–574, 1971.
36. LAMPKIN BC, NAGAO T, and MAUER AM. Synchronization of the mitotic cycle in acute leukemia. Nature 222:1274–1275, 1969.
37. WODINSKY I, SWINIARSKI J, and KENSLER CJ. Spleen colony studies of leukemia L1210. VII. Combined effects of hydroxyurea (NSC 32065) and γ-radiations on in vivo systems. Front Radiat Ther Oncol 4:79–92, 1969.
38. VALERIOTE F, and EGGERDING F. Time-dependent synergism with combination 5-fluorouracil (5FU) and X-radiation (X-R). Proc Am Assoc Cancer Res 12:64, 1971.

Approaches to Combination Chemotherapy in Rat, Mouse, and Hamster Tumors [1,2]

D. P. Griswold, D. J. Dykes, C. A. Kelley, B. J. Roberts, and C. A. Dominick [3]

Combination chemotherapy probably came into its widest recognition as a practical application of a theoretical concept when penicillin and streptomycin were given as a single entity to combat both gram-positive and gram-negative microorganisms. And that was not combination chemotherapy in the true sense of the word since each therapeutic moiety was directed at a different target population. In the treatment of cancer, the use of combinations of agents has become increasingly popular probably, for more than any other reason, because of evidence at the clinical level of the failure of single-agent treatment and because of demonstrated successes with multiple-agent treatment, particularly among the leukemias. There are, of course, other good evidences of the efficacy of using combinations of chemotherapeutic agents in the treatment of cancers at the clinical as well as at the experimental level. Too often, however, the selection of agents for combination use has been based on rather hazy theoretical grounds. An alternative to the theoretical approach is empiricism, but the magnitude of the problem seems overwhelming when the number of variables is considered.

Our purpose in the studies mentioned in this paper was to make a beginning attempt to bring together potentially useful hypotheses and supporting experimental data that might add to our conceptual knowledge of combination chemotherapy.

MATERIALS

The animal tumor systems used in these studies were:

1. B16 melanoma: maintained in serial passage by subcutaneous (sc) implantation into C57BL/6 mice; used in experiments in BDF_1 ($C57 \times DBA_2$) mice.
2. C3H mammary carcinoma: maintained in serial passage and used in experiments by sc implantation into C3H mice; 16th transplant generation from a spontaneous tumor.
3. DMBA-induced [4] adenocarcinoma of the breast in female Sprague-Dawley rats.

Transplantable (sc) tumors of the Syrian (golden) hamster:

4. Plasmacytoma No. 1
5. Melanotic melanoma No. 1
6. Osteogenic sarcoma No. 1

The following chemotherapeutic agents were used: methyl-CCNU, cyclophosphamide, adriamycin, melphalan, NSC–45388, dromostanolone propionate, arabinosylcytosine (ara-C), hydroxyurea, progesterone, and estradiol. All agents were given intraperitoneally except adriamycin, dromostanolone propionate, progesterone, and estradiol, which were given sc.

EXPERIMENTAL STUDIES

Among the theoretical and practical considerations for combination chemotherapy we have examined only three where increased or greater-than-additive antitumor activity may be realized from the employment of two or more drugs in simultaneous or sequential combination. These are: (*a*) differential host tox-

[1] Supported by contracts NIH-71-2098 from the Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare (DHEW), and PH43-66-71 from the Division of Cancer Biology and Diagnosis, NCI, NIH, DHEW.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[3] Southern Research Institute, Birmingham, Ala.

[4] DMBA = 7,12-dimethylbenz(*a*)anthracene.

icity; (*b*) specific drug resistance; and (*c*) altered tumor cell kinetics.

The term differential host toxicity is meant to imply less-than-additive host toxicity because of real or presumed effects in normal tissues—principally in blood-forming elements and intestinal epithelium. A more direct explanation may be additive or greater-than-additive antitumor toxicity with no increased toxicity to the host.

An example of this phenomenon may be seen in figure 1. Fragments of B16 melanoma were implanted sc into BDF_1 mice, and the mice were given methyl-CCNU, cyclophosphamide, or a simultaneous combination 2 days later. Results were based on the difference in time for treated (T) or control (C) tumors to reach a predetermined median tumor weight and were expressed as T–C in days. Data from our total experience (three to eight experimental groups per dose level) with each of the single agents were plotted against the fraction of a single dose LD10. The curves through those points were visually determined. Similarly derived results from the treatment of mice with B16 melanoma with the simultaneous combination of methyl-CCNU and cyclophosphamide (also given on Day 2 only) were added for comparison. An LD10 was estimated from each of the combinations simply by adding the LD10 fractions of each agent (eg, methyl-CCNU, 16 mg/kg = ca 0.44 single-dose LD10; cyclophosphamide, 100 mg/kg = ca 0.33; so 0.44 + 0.33 = 0.77 LD10 for the combination). In addition, actual results from internal toxicity controls were shown for the combination-treated groups. None of the treatment combinations resulted in greater than an LD10 in those nontumor-bearing mice, but antitumor activity from the combinations exceeded that observed after treatment with an LD10 of either single agent.

This evidence of potentiating antitumor effect may be seen more clearly in the results from a single experiment (fig 2) where mice bearing sc implants of B16 melanoma fragments were treated with cyclophosphamide every day for 1–15 days after tumor implantation, with methyl-CCNU on Days 1, 5, 9, and 13, or with the combination of the two given

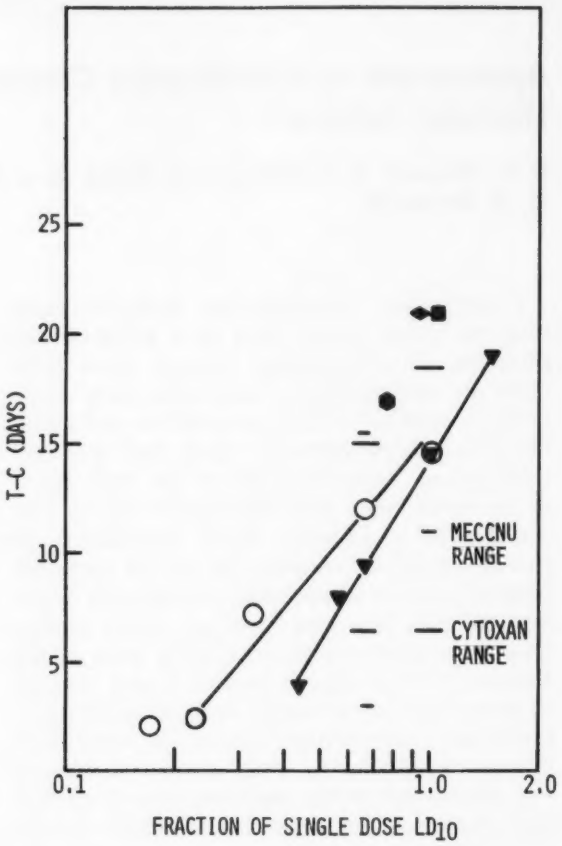

FIGURE 1.—Response of sc implanted B16 melanoma to methyl-CCNU, cyclophosphamide, or combinations of the two agents.

▼ = Methyl-CCNU: Day 2 only, total experience.
o = Cyclophosphamide: Day 2 only, total experience.
■ = Methyl-CCNU (36 mg/kg, Day 2 only) + cyclophosphamide (45 mg/kg, Day 2 only). Combination = > 1.0 LD10; 90% toxicity control survivors.
◆ = Methyl-CCNU (16 mg/kg, Day 2 only) + cyclophosphamide (100 mg/kg, Day 2 only). Combination = 0.77 LD10; 100% toxicity control survivors.
⬢ = Methyl-CCNU (7.3 mg/kg, Day 2 only) + cyclophosphamide (225 mg/kg, Day 2 only). Combination = 0.95 LD10; 100% toxicity control survivors.

simultaneously. When cyclophosphamide was given alone, only minimal antitumor activity was observed even at an LD30 dose (deter-

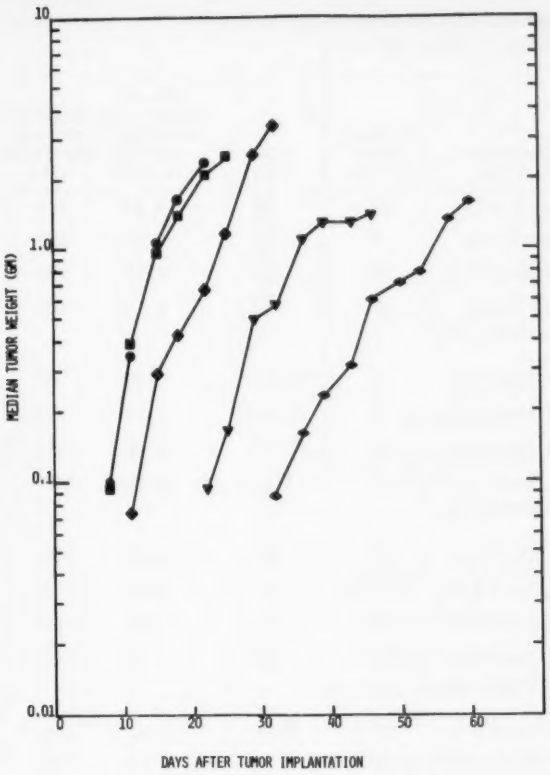

FIGURE 2.—Response of sc implanted B16 melanoma to methyl-CCNU, cyclophosphamide, or combinations of the two agents.

◆ = Cyclophosphamide: 24 mg/kg/day, qd Days 1–15. ILS = 55% = LD30.

■ = Cyclophosphamide: 6 mg/kg/day, qd Days 1–15. ILS = 23%.

▼ = Methyl-CCNU: 24 mg/kg/day, Days 1, 5, 9, 13. ILS = 120%.

◆ = Cyclophosphamide (6 mg/kg/day, qd Days 1–15) + methyl-CCNU (24 mg/kg/day, Days 1, 5, 9, 13). ILS = 173% = LD10.

● = Control.

mined from nontumor-bearing internal toxicity controls) under the chronic schedules employed; that treatment resulted in a T–C of about 7 days and a 55% increase in lifespan (ILS) of the host. When given alone the dose of cyclophosphamide used in the combination (6.0 mg/kg/day) resulted in no measurable T–C and only a 23% ILS. When added to methyl-CCNU, however, the T–C increased to 33 days compared with 17 days for methyl-CCNU alone. The ILS also reflected an increased antitumor activity. Although the dose of methyl-CCNU (24 mg/kg/dose, on Days 1, 5, 9, and 13) resulted in no deaths (LD0) in this trial, that dose is the historical LD10 in BDF_1 mice in our laboratories. The addition of cyclophosphamide (6.0 mg/kg/day, qd for 1–15 days) to methyl-CCNU resulted in an LD10 in the internal toxicity control.

Additional examples may be seen in table 1, where combination treatment resulted in greater T–C and ILS values and greater numbers of long-term tumor-free survivors (with no increased host toxicity) than resulted from single-agent treatment at maximally tolerated doses (LD10). An antitumor potentiating effect by combinations of drugs is not restricted to a single drug-pair nor to a single tumor system. The effect was seen with methyl-CCNU plus adriamycin in B16 melanoma, methyl-CCNU plus melphalan in melanotic melanoma No. 1, methyl-CCNU plus cyclophosphamide in osteogenic sarcoma No. 1, and adriamycin plus NSC-45388 in C3H breast carcinoma.

In a somewhat similar vein we have explored the effects of adding a cytotoxic agent to a course of androgen treatment in rats bearing estrogen-dependent tumors—a situation where additive host toxicity should be expected to be nil or negligible.

The DMBA-induced breast tumor in the female Sprague-Dawley rat has been reported to be hormone (estrogen) dependent, although the dependence is lost with increasing age. The tumors may be regressed by treatment with dromostanolone propionate but that regression is incomplete and transient (1). In figures 3–5, results are shown from three experiments where methyl-CCNU, melphalan, or cyclophosphamide was given alone or concomitantly with dromostanolone propionate to rats bearing DMBA-induced breast tumors.

Rats whose total tumor mass was 0.5 g each were taken from a pool of tumor-bearing rats and randomly distributed between treatment and control groups. Not more than 120 days had elapsed from the time of DMBA expo-

TABLE 1.—Summary of increased antitumor activity obtained with combination chemotherapy

Tumor	Agent	Dose level (mg/kg/dose)	Schedule (days)	Tumor delay (T-C)	ILS (%)	No. of tumor-free survivors/ total No. treated	Toxicity control survivors (%)
B16 melanoma	Methyl-CCNU	40	Day 2 only	22	33	2/10	100
	Methyl-CCNU	20	Day 2 only	6	25	0/10	100
	Adriamycin	2.2	qd, Days 2–9	18	120	2/10	100
	Methyl-CCNU	20	Day 2 only	27	149	4/10	100
	Adriamycin	2.2	qd, Days 2–9				
Melanotic melanoma No. 1	Methyl-CCNU	4.0	qd, Days 2–8	4	(0/10)*	0/10	80
	Methyl-CCNU	1.8	qd, Days 2–8	3	(0/10)*	0/10	90
	Melphalan	2.0	qd, Days 2–8	26	(5/10)*	0/10	90
	Methyl-CCNU	1.8	qd, Days 2–8	35	(7/10)*	0/10	90
	Melphalan	2.0	qd, Days 2–8				
Osteogenic sarcoma	Cyclophosphamide	22	qd, Days 2–8	31	82	1/10	100
	Methyl-CCNU	9.0	qd, Days 2–8	15	10	0/10	90
	Methyl-CCNU	4.0	qd, Days 2–8	6	5	0/10	80
	Cyclophosphamide	22	qd, Days 2–8	65	98	4/10	100
	Methyl-CCNU	4.0	qd, Days 2–8				
C3H breast carcinoma	Adriamycin	2.2	qd, Days 1–8	17	59	1/10	100
	Adriamycin	1.5	qd, Days 1–8	12	0	0/10	100
	NSC-45388	100	qd, Days 1–8	18	54	0/9	100
	NSC-45388	67	qd, Days 1–8	15	73	0/10	100
	Adriamycin	1.5	qd, Days 1–8	50	>170	4/10	100
	NSC-45388	67	qd, Days 1–8				

*90-day survivors.

sure to the initiation of treatment (experimental Day 1). Drug dose levels and treatment schedules may be seen in the legends to the figures. All rats were examined for tumors which were measured by caliper once a week. A responder was any rat whose total tumor mass regressed by 50% or more; such rats were considered to be in "remission." As previously mentioned, the treatment of rats with dromostanolone propionate will cause regression of their hormone-dependent breast tumors, but upon cessation of treatment the regressed tumors recur. It was the purpose of these trials to determine whether the addition of a cytotoxic agent to the androgen treatment regimen would delay or prevent the anticipated recurrence. When methyl-CCNU, melphalan, or cyclophosphamide was given intermittently during the dromostanolone propionate treatment period, the percents in remission were 44%, 50%, and 50%, respectively, compared to the dromostanolone propionate control values which were zero, 13%, and 28%. Host deaths after treatment with methyl-CCNU, melphalan, or cyclophosphamide were 40%, 20%, and 40%, respectively; but when the same agents were given in combination with dromostanolone propionate, host deaths were 10%, 10%, and 5%, respectively. Thus, it appears that both increased antitumor activity and decreased normal tissue toxicity resulted from use of those combinations.

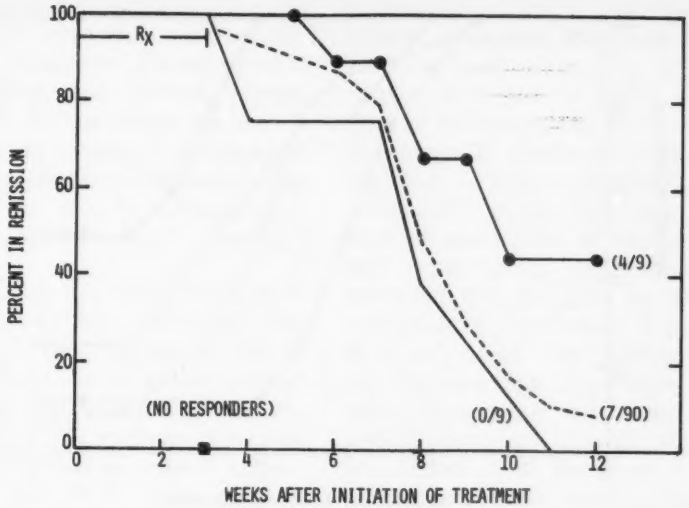

FIGURE 3.—Response (measured in percent remission) of rats bearing DMBA-induced tumors treated with dromostanolone propionate (10 mg/kg/day, qd Days 1–20).

—— = Internal control.

..... = Total experience.

■ = Methyl-CCNU: 12 mg/kg/day, Days 1, 8, 15. No responders.

● = Combination of dromostanolone propionate and methyl-CCNU.

( ) = No. of animals in remission at end of experimental period/ No. in remission at end of treatment.

A second category for consideration in the selection of agents for combination use is that of specific drug resistance. Both resistance and cross-resistance of bacterial systems and experimental tumor systems to a large number and variety of agents have been reported (2). The data in table 2 show the basis for our concern about the possibility of resistance of the B16 melanoma to methyl-CCNU. Although these results came from different experiments, the results from treating the mice one or four times at 24 mg/kg/dose were total experience data (seven to nine experiments each) and were therefore satisfactory for measurement of order-of-magnitude differences. When 36 or 33 mg/kg was given once or twice, the calculated T–C per dose was the same, 15 days. When 24 mg/kg was given one or four times, the calculated T–C per dose was the same, 9.0 days, only if it was assumed that only two instead of four doses of methyl-CCNU were given. These results suggested that methyl-CCNU resistance became detectable and significant after the second dose of methyl-CCNU.

In a separate series of experiments (fig 6), we set out to develop a methyl-CCNU-resistant subline of B16 melanoma. Tumor fragments from our regular stock passage line, a line repeatedly shown to be sensitive to methyl-CCNU and designated B16/0, were implanted sc by trocar into 20 mice subsequently divided into two groups. One group was given a single dose of methyl-CCNU, 36 mg/kg, 2 days after implantation. The other group was held as a control. When tumors in the group treated with methyl-CCNU reached approximately 1.0 g, fragments from one of those tumors were transplanted to an additional 20 mice, one half of which were treated with methyl-

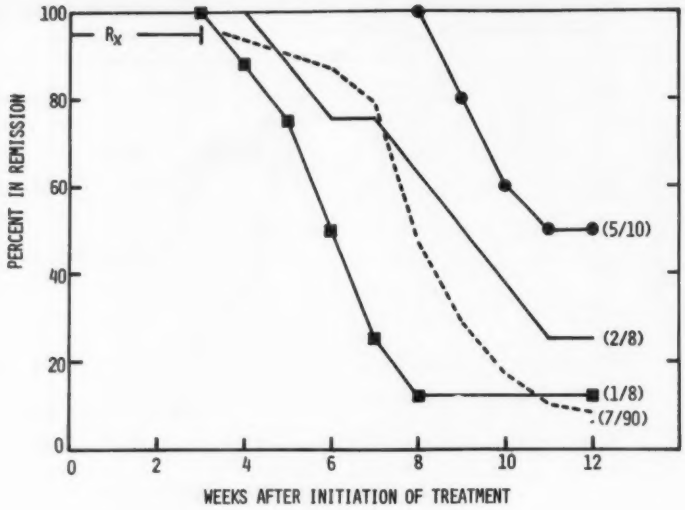

FIGURE 4.—Response (measured in percent remission) of rats bearing DMBA-induced breast tumors treated with dromostanolone propionate (10 mg/kg/day, qd Days 1–20).

—— = Internal control.

. . . = Total experience.

■ = Melphalan: 6.0 mg/kg/day, Days 1, 8, 15.

● = Combination of dromostanolone propionate and melphalan.

( ) = No. of animals in remission at end of experimental period/ No. in remission at end of treatment.

TABLE 2.—Comparison of the response of sc implanted B16 melanoma to single- and multiple-dose methyl-CCNU treatment

Dose level (mg/kg/dose)	No. of treatments	Interval between treatments (days)	Total interval (days)	T–C * (days)	Adjusted T–C † (days)	Adjusted T–C ‡ (days)	T–C/ treatment
24§	1	0	0	9.0	—	—	9.0
24§	4	4	12	22	10	18	(9.0)
36¶	1	0	0	15	—	—	15
33**	2	7	7	37	30		15

*T–C = difference in days between control and treated time to appearance of median tumor weight to 0.5 g.
†Total T–C less interval between first and last treatments.
‡Total T–C less interval between first and second treatments, assuming no effect from last 2 treatments.
§Median values from 7 experiments.
¶Median values from 9 experiments.
**Single experiment.

CCNU as before. That procedure was continued through several passages. Tumor-growth curves of both treated and control groups from each of the first four passages are shown in figure 6. On the third passage and exposure to methyl-CCNU, tumors were markedly less responsive, and by the fourth passage they were completely unresponsive to methyl-CCNU.

When mice bearing tumors from that resistant line (B16/methyl-CCNU) were treated with cyclophosphamide (133 mg/kg on Day 2 only), they were as sensitive as B16/0 to that agent, suggesting no cross-resistance (fig 7). Thus, the combination of cyclophosphamide plus methyl-CCNU may be considered appropriate not only because of less-than-additive host toxicity but because of the opportunity to overcome specific drug resistance as well.

The third category for consideration involves a tumor population growth characteristic where the possibility exists for a favorable kinetic alteration. We have previously shown (3) that reduction of a significant fraction (about 99%–99.9%) of the clonogenic population of plasmacytoma No. 1 (pla 1) by cyclophosphamide treatment results in a transient increase in growth fraction of the surviving tumor cell population and, thereby, an increased sensitivity to the cell-cycle-stage-specific agent, ara-C. The results of a similar experiment (fig 8) point out the increased sensitivity of a population of plasmacytoma cells to ara-C when that population was reduced by exposure to a single dose of cyclophosphamide. When ara-C was given to previously untreated hamsters bearing pla 1, only a minimal delay (2–3 days) in tumor recurrence was observed, but when ara-C treatment followed cyclophosphamide, a significantly greater delay, above that observed following cyclophosphamide alone, was seen in tumor recurrence, and seven of 20 tumors failed to recur. In this instance, increased sensitivity of the

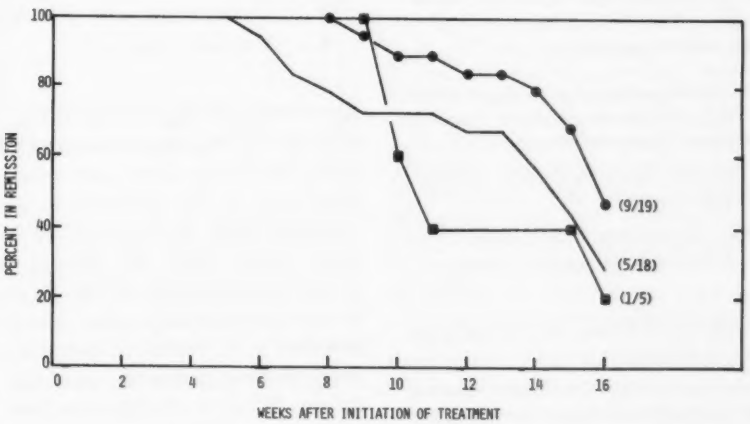

FIGURE 5.—Response (measured in percent remission) of rats bearing DMBA-induced breast tumors.

—— = Dromostanolone propionate: 10 mg/kg/day, 3 times weekly for 10 wks. Eighteen of 19 animals responded.

■ = Cyclophosphamide: 60 mg/kg/day, Days 1, 13, 25, 37. Five of 19 animals responded.

● = Dromostanolone propionate (10 mg/kg/day, 3 times weekly for 10 wks) + cyclophosphamide (60 mg/kg/day, Days 21, 33, 45, 67). Twenty of 20 animals responded.

( ) = No. of animals in remission at 16 wks/No. in remission at end of treatment.

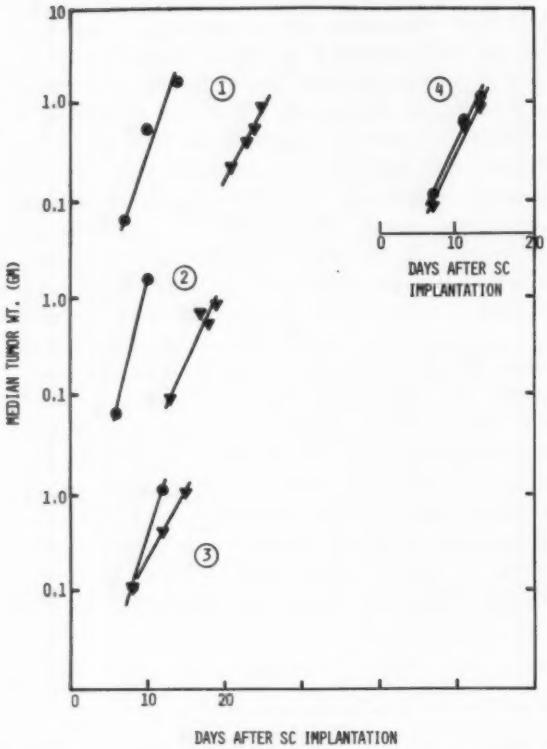

FIGURE 6.—History of development of a methyl-CCNU-resistant line of B16 melanoma. (Circled numbers indicate No. of passages from sensitive line.)

● = Untreated control from previous methyl-CCNU-treated group.

▼ = Methyl-CCNU: 36 mg/kg, Day 2 only. From previous methyl-CCNU-treated group.

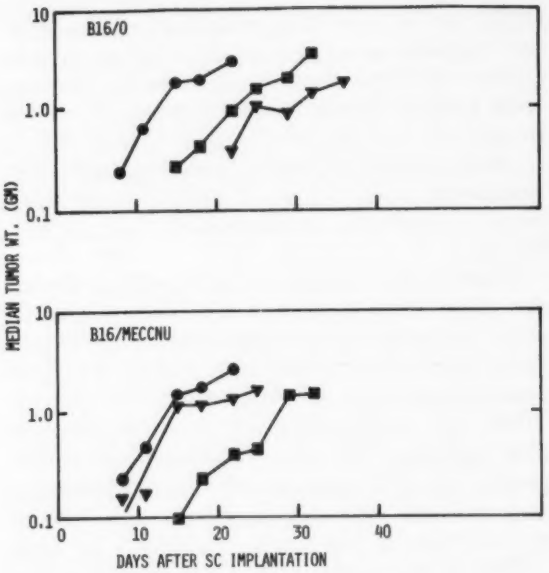

FIGURE 7.—Response to methyl-CCNU or cyclophosphamide of methyl-CCNU-sensitive (B16/0) and -resistant (B16/methyl-CCNU) lines of B16 melanoma.

▼ = Methyl-CCNU: 36 mg/kg, Day 2 only.

■ = Cyclophosphamide: 133 mg/kg, Day 2 only.

● = Untreated control.

tumors to ara-C was an indirect result of pretreatment with the cytotoxic agent.

In an alternative scheme, we have shown that a tumor cell population may be directly stimulated in order to increase its sensitivity to a cycle-specific agent. When rats bearing DMBA-induced tumors are ovariectomized at appropriate ages, their tumors regress and remain regressed (in a state of remission) for varying periods of time (1). However, when those rats in a state of remission are given progesterone and estradiol, their tumors grow rapidly, and the thymidine index has been reported to increase by about tenfold (4). The results of an experiment based on that phenomenon are shown in table 3. Hydroxyurea was given to rats bearing DMBA-induced tumors at a dose level and schedule (40 mg/kg/dose, q6h × 7) thought to be appropriate in consideration of the cell-cycle parameters of that tumor type (4). One group of rats was given hydroxyurea alone. A second group was given hydroxyurea after their tumors had regressed as a result of ovariectomy and during progesterone/estradiol-induced growth stimulation. When hydroxyurea was given to otherwise untreated, tumor-bearing rats no reduction in median tumor weight, mean tumor number, or percent of rats with tumors was apparent from comparison with an untreated control. Hydroxyurea treatment of rats bearing tumors whose growth rates were under the influence of progesterone and estradiol resulted in markedly smaller median tumor weight, mean tumor number, and percent of rats with tumors than observed after tumor growth stimulation without hydroxyurea.

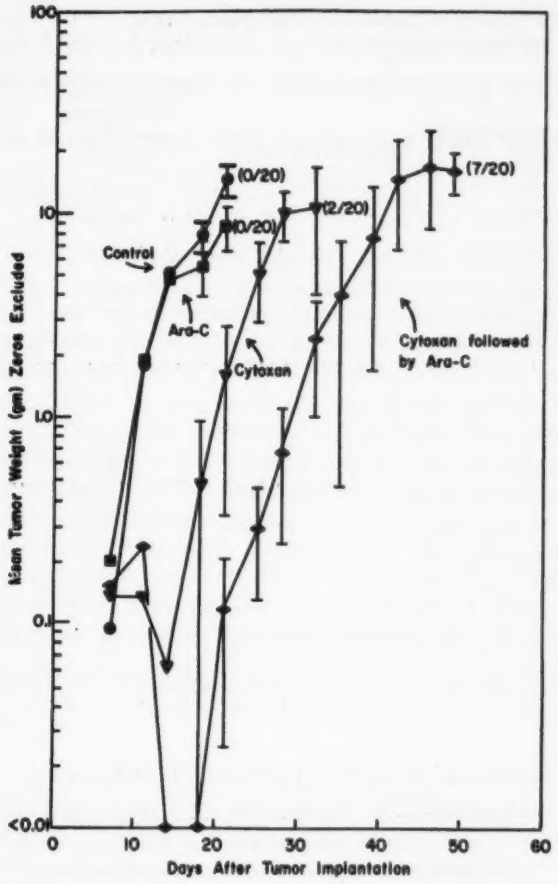

FIGURE 8.—Comparison of response of plasmacytoma No. 1 to arabinosylcytosine (ara-C) with and without previous cyclophosphamide treatment. Mean tumor weight plotted to 50% host mortality.
Cyclophosphamide: 30 mg/kg/dose, Day 7 only.
Ara-C: 10 mg/kg/dose, q3h × 8, Days 12, 16, 20, 24.
I = One standard deviation from the mean.

DISCUSSION

The selection of agents for use in combination therapy regimens is, of necessity, in large part empirical at this time because of our lack of detailed knowledge of:

1. the specific mechanism of action of individual agents at the subcellular level;
2. the mechanism, timing, and control of cell repair;
3. the development of drug resistance at the cell level;
4. metabolic and proliferative rates of very small tumor cell populations; and
5. the interrelationship between chemotherapeutic activity and the host immune mechanism.

Nevertheless, the rationales for the use of chemotherapeutic agents in simultaneous or sequential combination are increasing. We have presented here only limited experimental data attesting to the validity of three concepts. The first concept, differential host toxicity, may be expected to have wide applicability to a large variety of tumors in man. The second, specific drug resistance, may be of more limited usefulness although clinical attention to this problem has been reported (5). The third consideration, altered tumor cell kinetics, will probably be as unique in man as it has been in experimental trials, but the rationale has had clinical consideration (6).

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Arabinosylcytosine: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C; cytosine arabinoside

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Dromostanolone propionate: NSC-12198; 5α-androstan-3-one, 17β-hydroxy-2α-methyl-, propionate; 2-α-methyldihydrotestosterone propionate; MDTP

Estradiol: NSC-9895; CAS reg. No. 50-29-2

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-sarcolysin

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

NSC-45388: CAS reg. No. 4342-03-4; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DIC

Progesterone: NSC-9704; CAS reg. No. 57-83-0; pregna-4-ene-3,20-dione

REFERENCES

1. GRISWOLD DP, JR, and GREEN CH. Observations on the hormone sensitivity of 7,12-dimethylbenz(α) anthracene-induced mammary tumors in the Sprague-Dawley rat. Cancer Res 30:819–826, 1970.
2. SKIPPER HE, HUTCHISON DJ, SCHABEL FM, JR, ET AL. A quick reference chart on cross resistance between anticancer agents. Cancer Chemother Rep 56:493–498, 1972.
3. GRISWOLD DP, JR, SIMPSON-HERREN L, and SCHABEL

TABLE 3.—Response of DMBA-induced breast tumors in female Sprague-Dawley rats to hormonal alteration and cytotoxic agent treatment

Agent or treatment	Dose and schedule	Median tumor weight (g)	Mean tumor No.	No. of survivors with tumors/ total No. treated
Hydroxyurea	40 mg/kg/dose, q6h × 7, Days 0, 5, 10, 15	3.6 * (2.2 †	3.8 2.0	10/10 10/10)
Ovariectomy	—	1.9	1.8	8/10
Progesterone	8.0 mg/rat/day, qd, Days 31–60			
Estradiol	2.0 μg/rat/day, qd, Days 31–60			
Ovariectomy	—	0.05	1.1	4/8
Progesterone	8.0 mg/rat/day, qd, Days 31–60			
Estradiol	2.0 μg/rat/day, qd, Days 31–60			
Hydroxyurea	40 mg/kg/dose, q6h × 7, Days 33, 38, 43, 48			

*Results computed on Day 63.
†Results computed on Day 21.

FM, JR. Altered sensitivity of a hamster plasmacytoma to cytosine arabinoside (NSC-63878). Cancer Chemother Rep 54:337–346, 1970.

4. SIMPSON-HERREN L, and GRISWOLD DP, JR. Studies of the kinetics of growth and regression of 7,12-dimethylbenz(α)anthracene-induced mammary adenocarcinoma in Sprague-Dawley rats. Cancer Res 30:813–818, 1970.
5. BERGSAGEL DE, COWAN DH, and HASSELBACK R. Plasma cell myeloma: response of melphalan-resistant patients to high-dose intermittent cyclophosphamide. Can Med Assoc J 107:851–855, 1972.
6. SALMON SE, MCINTYRE RO, and OGAWA M. IgE myeloma: total body tumor cell number and synthesis of IgE and DNA. Blood 37:696–705, 1971.

In Vitro Methods to Complement In Vivo Approaches Utilizing Combinations of Chemotherapy and Immunotherapy

M. Michael Sigel, Wolf Lichter, and Diana M. Lopez [1]

SUMMARY

Cell cultures have already proven their worth in measuring the cytotoxic and cytostatic effects of many synthetic drugs as well as drugs derived from natural products. New methods available for the study of cytokinetics and cellular morphology should be added to the existing procedures for the evaluation of actions of single drugs and drug combinations applied at one time or in sequence. Lymphocyte functions amenable to measurements in vitro have provided useful criteria for the assessment of the immunologic status of animals and patients undergoing chemotherapy. Greater use should be made of in vitro methods employing lymphocytes. The blastogenic responses of lymphocytes as well as the cytotoxic functions of these cells should be exploited for measurement of drug action in vitro, thereby providing in vitro parallels for in vivo studies.

[Cancer Chemother Rep Part 2, vol 4:109–114, 1974]

A considerable amount of primary screening of compounds with anticellular activity is performed in cell cultures. The long-established KB cell culture system has proved valuable for this purpose although other cells derived from tumors have found favor with some investigators. Under the auspices of Drug Research and Development, National Cancer Institute, National Institutes of Health, work is being launched with L1210 and P388 leukemia cell cultures as supplements to the KB screen. This should permit the direct comparisons of in vivo and in vitro procedures with leukemic cells with respect to many parameters of experimental chemotherapy and immunotherapy. Cell cultures are especially useful for initial detection of activities in crude extracts of natural products and for measurement of activities in components derived from various fractionation procedures, especially where only small amounts of material are available. Data from such in vitro screening systems are rapidly acquired (3–4 days) and are highly reproducible. The tests are relatively inexpensive and simple, which makes it possible to perform large-scale determinations.

While these attributes of the in vitro screen make it a highly useful procedure, the drug screener must be aware of its limitations. Cells which are removed from their anatomic associations and natural milieu lack the attenuative relief afforded by the body's catabolic diffusional and sequestering machinery, and are more vulnerable to drug action. Also, there is the reverse situation, where the drug is inert in its native form and must be converted to an active moiety through enzymatic action. We are all familiar with the classic case of cyclophosphamide,[2] which is active in vivo but inactive in cell cultures. Another deficiency of the existing in vitro chemotherapeutic assays

[1] Laboratory of Virology, University of Miami School of Medicine, Fla.

[2] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

is that they do not take into account the potentially favorable or adverse effects which would accrue from the impact of the drug on the immune mechanism. We hope to develop some ideas which, if successfully implemented, will make the cell culture capable of measuring such indirect effects.

Cell cultures have already proved to be of immeasurable value in studies dealing with problems of cellular kinetics, mutants with altered susceptibility to drug action, and the relationships of drug effectiveness to the phase of the cellular growth cycle. Clear-cut evidence for the heterogeneity of the tumor population has been furnished by experiments with cell cultures (1, 2). Thus, four clones derived from a single human melanoma were found to vary in their response to BCNU and cytosine arabinoside.[3] As shown in figure 1, clone 19-4 was most resistant and clone 39-5 was highly susceptible to BCNU. A reversed relationship was noted in cytosine arabinoside; clone 39-5 was totally resistant and clone 19-4 showed maximum susceptibility, albeit only on the order of about 50% cell kill. These results were obtained with nonsynchronized cultures. Experiments with synchronized cultures have provided important information on the relationship of drug action to a particular phase of the cell cycle. This is depicted in figure 2 where the maximal effect of bleomycin (NSC-125066) is exerted on the cells in mitosis (3).

[3] BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

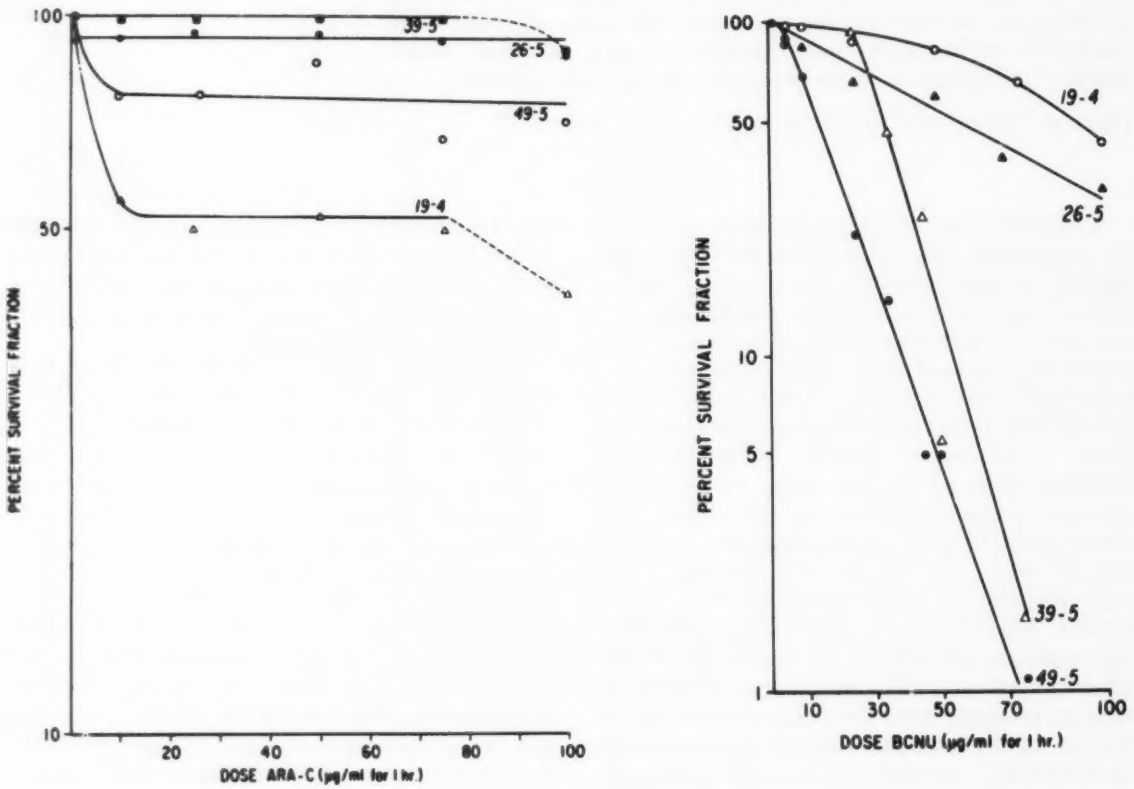

FIGURE 1.—Effects of cytosine arabinoside and BCNU on four clones of human melanoma cells derived from a single tumor. (*Left*: Reproduced with permission from ref 1. *Right*: Reproduced with permission from ref 2.)

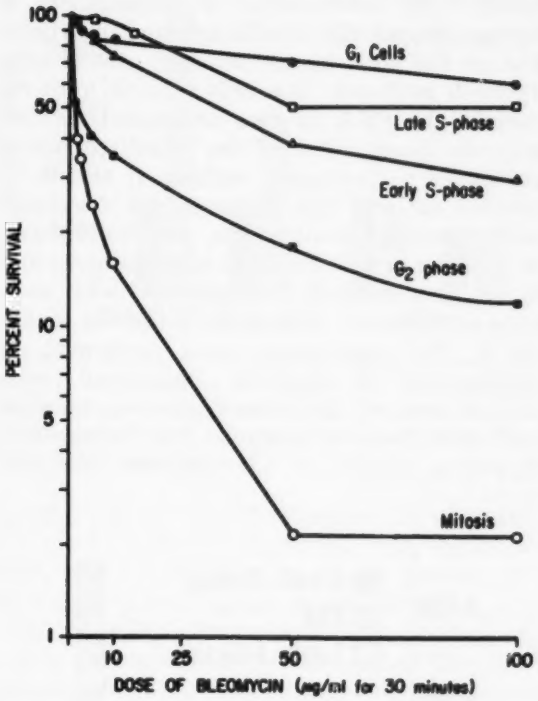

FIGURE 2.—Effect of bleomycin on Chinese hamster cells in various stages of the cell cycle. (Reproduced with permission from ref 3.)

Let us now consider the capabilities and potentials of cell culture methods for assessing the effects of combined therapy and the interaction of immunologic parameters with chemotherapy.

Synergistic Activities

Heretofore, cell cultures have been used for measuring cytotoxic activities of single synthetic and natural products, their derivatives, and separated components. Yet, there are indications that the system could be adapted to the exploration of synergistic activities. Reference has already been made to the use of synchronized cultures for the delineation of specificity of activity in relation to phases of the cell cycle. But even unsynchronized cultures could be used for the measurement of potentiating effects of one drug upon the other with the drugs added simultaneously or in sequence. As in the case of in vivo combined therapy trials, the drugs could be used in graded doses below the ED50 previously established in single-agent assays. In this type of work the ED50 which defines the significance of growth inhibition may not suffice. It may be necessary to design criteria other than the quantification of protein as an index of cell growth on Day 3. Investigators at A. D. Little, Inc. (Cambridge, Mass) have been using a procedure which measures the inhibition of colony formation in soft agar (4). This technique can reveal subtle alterations in the cell's viability which may be unnoticed in mass cultures. In order to obtain representative samples of cells at different time intervals in relation to the introduction of one or more drugs, it may be essential to perform such tests in spinner cultures utilizing dispersed cell populations. Assessment of cell damage can be accomplished through any of the following procedures: (*a*) microscopic examination of wet mounts and of stained smears could be used to demonstrate gross morphologic changes; (*b*) differential uptake of dyes would serve as a further indicator of cell damage; (*c*) prelabeling of the cells with radioisotopes is another technique which provides a quantitative assay since one can measure either the amount of label remaining in the cells or the amount of label released into the supernatant fluid. These methods have proved very useful for assaying immune cytotoxicity, and we will say more about this later.

We are currently investigating the utility of an automated device, the Cytograf (Biophysics Systems, Inc., Mahopac, NY), which is capable of measuring three parameters: the numbers of cells, cell size, and cell viability. The latter is based on the ability of the laser beam to discriminate between unstained and stained cells utilizing trypan blue. We have as yet no definitive results because the machine has just recently been installed. But the studies by H. Rothschild (personal communication) have provided data indicating that the Cytograf is valuable in studies on the kinetics of drug action.

Lymphocyte Response in Tissue Culture

One of the in vitro reactions which appears to be relevant to the state of cellular immunity

is the lymphocyte response to stimuli in tissue culture. Generally speaking, lymphocytes are in phase G_0 until stimulated toward cell division. Such stimulation can be brought about by specific antigens or by nonspecific mitogens such as phytohemagglutinin (PHA), concanavalin A, or bacterial lipopolysaccharide (LPS). The first two are said to stimulate T-cells while LPS apparently stimulates B-cells. All lymphocytes are derived from stem cells in bone marrow. Some migrate to the thymus and from there to the peripheral lymphoid organs. These are the T-cells which are concerned mainly with cell-mediated immunity, including cytotoxicity. Other descendants of the stem cells bypass the thymus on their way to the lymphoid organs. These are the B-cells whose main function is antibody synthesis and secretion. However, the T-cells and B-cells cooperate in antibody productive response through a mechanism(s) not yet fully elucidated. Furthermore, there are indications that B-cells may also exert cytotoxic effects. For more information on these subjects, see references 5–10.

There are many tests available for the measurement of lymphocyte stimulation by antigens and mitogens. They differ mainly in the manner in which the response is determined. One criterion of response is the appearance of enlarged lymphocytes (blast cells), hence the overall name for this set of procedures is the blastogenic reaction. Another sign is a heightened rate of mitosis which can be seen by the increased frequency of cells in metaphase. However, both methods are tedious and not very quantitative. Hopefully, the new instruments entering the field, such as the Cytograf, may refine the method through automation and greater precision. At present, the more quantitative methods are based on physicochemical procedures which measure the incorporation of radioactive precursors as an index of biosynthetic processes which precede cell division. Thus, one can use tritiated thymidine to measure DNA synthesis or radioactive amino acids to assay protein synthesis. In our laboratory we have been studying lymphocyte responses to a variety of mitogens and specific antigens including tumor antigens. Figure 3 illustrates thymidine uptake by spleen cells from a normal mouse and from a mouse sensitized to a transplantable mammary tumor. The incorporation of radioactivity is always compared to the background incorporation of the label in unstimulated control cells (growth medium). The cells of both mice responded to PHA in two concentrations but only the lymphocytes of the sensitized mouse responded to the tumor antigen. It should be pointed out that this tumor is not associated with mammary tumor virus, and the antigen in question is not related to viral antigens and is not virus-induced. Data obtained with antigens of oncogenic viruses are presented in figure 4. The experiments were performed on lymphocytes of chickens immunized with RAV-1 virus of the leukosis-sarcoma complex and nonimmunized controls. The background thymidine uptake is low (growth medium)

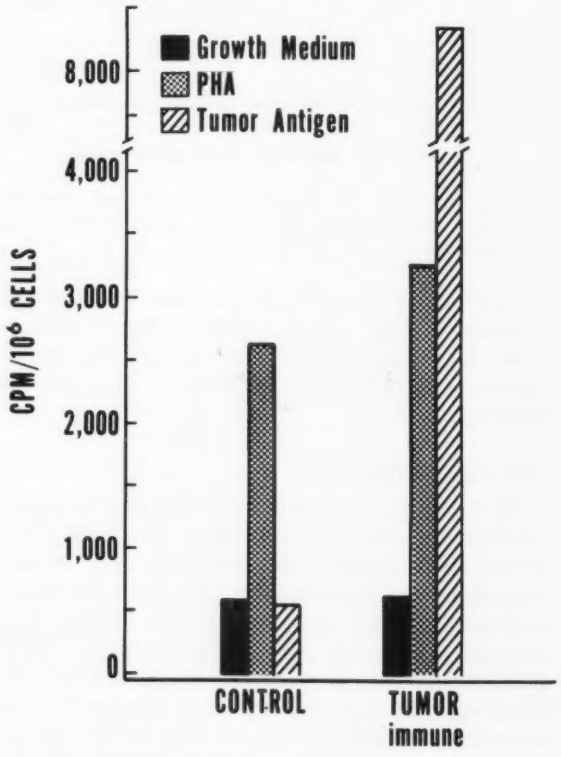

FIGURE 3.—Blastogenic response measured by increased thymidine uptake in mouse spleen cells stimulated by PHA or tumor antigen (data obtained from experiments by Lopez DM, Meyers P, and Sigel MM).

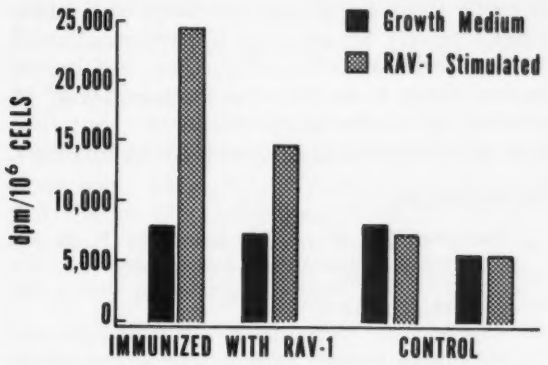

FIGURE 4.—Blastogenic response measured by increased uptake of thymidine in peripheral leukocytes of chickens immunized with leukosis virus and control chickens (based on experimental data of Lopez DM, Sigel MM, and Meyers P).

and the antigen (RAV-1) causes no stimulation in the control (unimmunized) lymphocytes, but it stimulates a higher uptake of thymidine in the immune lymphocytes.

This procedure makes possible the testing of several parameters of drug action. The test can measure drug-induced immunosuppression as well as the recovery from the immunosuppressed state. It can assess the changes in lymphocyte reactivity to tumor antigens (which is the crux of the problem), non-tumor antigens relevant to tumor immunity (eg, the antigens of the tubercle bacillus [PPD] if the patient is receiving bacillus Calmette-Guérin [BCG] as an immunotherapeutic modality), as well as irrelevant antigens and mitogens. Studies with antigens of the last-mentioned category have been conducted by investigators at the M. D. Anderson Hospital and Tumor Institute (Houston, Tex) and at Baylor Medical College (Waco, Tex) (11). They compared immunosuppressive effects of continuous and intermittent chemotherapy on immunization with hemocyanin. The antibody response was diminished more severely by intermittent than by continuous therapy. The immediate impression created by these results was that continuous therapy is superior to intermittent therapy, but this is not necessarily so if one evaluates the data in the context of current concepts of the operation of the several components of the immune response. Although antibodies may exert cytotoxic effects under certain conditions, certain antibodies (or antigen-antibody complexes) are known to block cell-mediated immune functions, thereby shielding the tumor and allowing it to progress. Thus, reduction of antibody synthesis may be a welcome consequence of chemotherapy, but this whole problem requires considerably more investigation, both in vivo and in vitro, as it has direct relevance to cancer immunity. In the same investigations, lymphocyte responses measured by the incorporation of thymidine were considerably less impaired by intermittent than by continuous chemotherapy, which indicates that the former was less deleterious to the integrity of the defense mechanism.

In the studies just described, the drug action was exerted in vivo and the measurement of one aspect of lymphocyte competence was accomplished in vitro. Both aspects can now be studied in cell cultures. Many different experiments can be designed and we would like to mention a few. The lymphocytes could be taken from tumor-bearing or tumor-immune animals or from cancer patients. Single or multiple drugs may be interposed before the application of a stimulant and then washed away. In this way, one can ascertain whether the drug(s) is capable of blocking or destroying the receptor site which would otherwise bind the immunologic stimulant. In another type of experiment the drugs can be added after the application of the stimulant and the events followed to determine whether the drug(s) prevents DNA synthesis or other aspects of the blastogenic process. The stimuli can include tumor cells (however, because of the possible interplay of histocompatibility antigens, intact cells may be restricted to autologous systems, a point requiring further investigation), tumor cell extracts, PPD (if the lymphocytes originated from patients or animals undergoing BCG immunotherapy), PHA, etc. Antibodies to the stimuli are known to influence the lymphocyte response, usually by blocking it in a manner similar to the blocking of cytotoxic activity of lymphocytes. The capability of a drug to relieve the antibody blocking effect could also be studied by these procedures. Finally, with the emerging new con-

cepts of the role of T-cells and B-cells in immunity (in general) and cancer immunity (in particular), one could design experiments which would measure the selective action of drugs on the response of T-cells and B-cells to their respective stimuli.

Immune Cytotoxic Reactions

Tumor cells can be destroyed either by antibody and complement or by immune lymphocytes. As stated above, the cell-mediated cytotoxicity can be blocked by antibodies. Damage to tumor cells can be assessed by means of microscopic observations or by means of assays which measure the release of radioactive chromium (^{51}Cr). Other methods and modifications have also been used but they need not concern us here. One of the commonly used procedures employs microtiter plates. The tumor cells are grown on the bottoms of the wells. Antibody and complement can bring about a significant effect in 1–2 hours. Cell-mediated toxicity requires a longer time interval. The extent of cytotoxic activity is measured by the amount of chromium released into the supernatant fluids. The effect of drugs can be assessed in a variety of ways. For example, one can pretreat tumor cells with single or multiple drugs and then expose them to the action of antibody and complement or immune lymphocytes in order to determine whether they are rendered more vulnerable. In this type of experiment one should employ both target cells which are free of antibody and target cells with "blocking" antibody attached to the surfaces. The reverse experiment would be to determine whether a drug(s), especially one known not to inhibit DNA synthesis, is able to purge lymphocytes of soluble antigen or antigen-antibody complexes as these two apparently inhibit cytotoxic reactions of lymphocytes.

Other cells or cellular functions are known to contribute to cancer immunity and may be affected by chemotherapeutic agents, but time does not permit us to go into these at this time.

REFERENCES

1. Barranco SC, Ho DHW, Drewinko B, et al. Differential sensitivities of human melanoma cells grown in vitro to arabinosylcytosine. Cancer Res 32:2733–2736, 1972.
2. Barranco SC, Drewinko B, and Humphrey RM. Differential response by human melanoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea and bleomycin. Mutat Res 19:277-280, 1973.
3. Barranco SC, and Humphrey RM. The effects of bleomycin on survival and cell progression in Chinese hamster cells in vitro. Cancer Res 31:1218–1223, 1971.
4. Himmelfarb P, Thayer PS, and Martin H. Growth of colonies of murine leukemia L1210 in vitro. Cancer Chemother Rep 51:451–453, 1967.
5. Good RA, and Finstad J. Relation between structure and function of the lymphoid systems. A developmental perspective. *In* International Convocation on Immunology (Rose NR, and Milgrom F, eds). Basel, Switzerland, S. Karger, 1969.
6. Miller JFAP, and Mitchell GF. Thymus and antigen-reactive cells. Transplant Rev 1:3–42, 1969.
7. Paul WE. Functional specificity of antigen-binding receptors of lymphocytes. Transplant Rev 5:130–166, 1970.
8. Niederhuber JE, and Möller E. Antigenic markers on mouse lymphoid cells: the presence of MBLA on antibody forming cells and antigen binding cells. Cell Immunol 3:559, 1972.
9. Hamburger J, and Bach J-F, eds. Cellular aspects of transplantation. *In* International Symposium on Aspects of Transplantation. Transplant Proc 4:157–284, 295–426, 1972.
10. Braun W. Regulation of the immune response in tumor immunology. *In* Virus Tumorigenesis and Immunogenesis (Ceglowski WS, and Friedman H, eds). New York, Academic Press, 1973.
11. Frei E, III. Combination cancer therapy. Presidential address. Cancer Res 32:2593–2607, 1972.

Biochemical Aspects of Drug Combinations

R. W. Brockman [1]

The objective of this presentation is to consider some examples of combination therapy and discuss briefly biochemical bases for this approach to chemotherapy. By way of introduction, information about the effects of some of the major classes of anticancer agents on the synthesis of macromolecules and on cell division will be discussed. Figure 1 summarizes some of the recognized actions of various agents on (*a*) the synthesis of purine and pyrimidine ribonucleotides, (*b*) the synthesis of deoxyribonucleotides from ribonucleotides, (*c*) the polymerization of nucleotides, (*d*) the DNA itself (eg, binding to DNA; covalent reaction with DNA), and (*e*) the process of mitosis. Agents that react with or bind to DNA may affect replication or repair of DNA or they may interfere with transcription of DNA to RNA which, in turn, may alter protein synthesis. It can be seen that agents such as glutamine antagonists (1), purine analogs (after conversion to nucleotides) (2,3), and folate analogs (4) inhibit the formation of purine ribonucleotides; the pyrimidine analog, 6-azauridine,[2] after phosphorylation to the nucleotide, blocks the synthesis de novo of pyrimidine ribonucleotides (5). The formation of deoxyribonucleotides is inhibited by ribonucleotide reductase inhibitors (hydroxyurea [6,7], guanazole [8], and heterocyclic aldehyde thiosemicarbazones [9–11]), by methotrexate (4), and by 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine after they are metabolized within the cell to 5-fluoro-2′-deoxyuridylic acid (12). Arabinosylcytosine (ara-C), after its intracellular conversion to arabinosylcytosine 5′-triphosphate, inhibits the synthesis of DNA (13,14). Thus, there are a number of different agents that inhibit many steps on the pathway to DNA by quite different mechanisms. As will be seen, this offers opportunities for using combinations of agents.

An interesting variety of agents act on DNA itself and thereby on the processes of replication, repair, and transcription of DNA. The actions of radiation (15,16) and of alkylating agents (17,18) on DNA are well known. In recent years a number of agents—both synthetic compounds and natural products—have been recognized that exert biologic effects by acting on DNA (19). These include agents that react with DNA, such as the nitrosoureas (20,21), *cis*-diamminedichloroplatinum (22), and mitomycin C (23). The chromomycin antibiotics, such as mithramycin (19), and the anthracycline antibiotics, such as daunorubicin and adriamycin (24), bind to DNA and affect polymerase activities. Bleomycin, a new antitumor antibiotic (25), binds to DNA and produces strand scission (26,27); this antibiotic was also reported to inhibit a DNA-dependent DNA polymerase from an oncogenic RNA virus (28). Agents such as the vinca alkaloids appear to act by interference with the process of mitosis (29).

One also must recognize that some purine and pyrimidine analogs may be incorporated into RNA or DNA. Thus, for example, 8-azaguanine (30) and 6-thioguanine (6-TG) (31) are incorporated into both RNA and DNA of tumor cells; 5-FU (12) is incorporated only into RNA. The incorporation of 6-TG into DNA is considered to be a significant factor in its antitumor effect (31). The incorporation of 8-azaguanine (32,33) and 5-FU (34) into cellular RNA fractions, particularly messenger RNA, may result in inhibition of protein synthesis or synthesis of altered proteins. Although, in the case of 5-FU, these effects are secondary to the inhibition of DNA synthesis, the incorporation of 5-FU into RNA may contribute to side effects.

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[1] Southern Research Institute, Birmingham, Ala.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

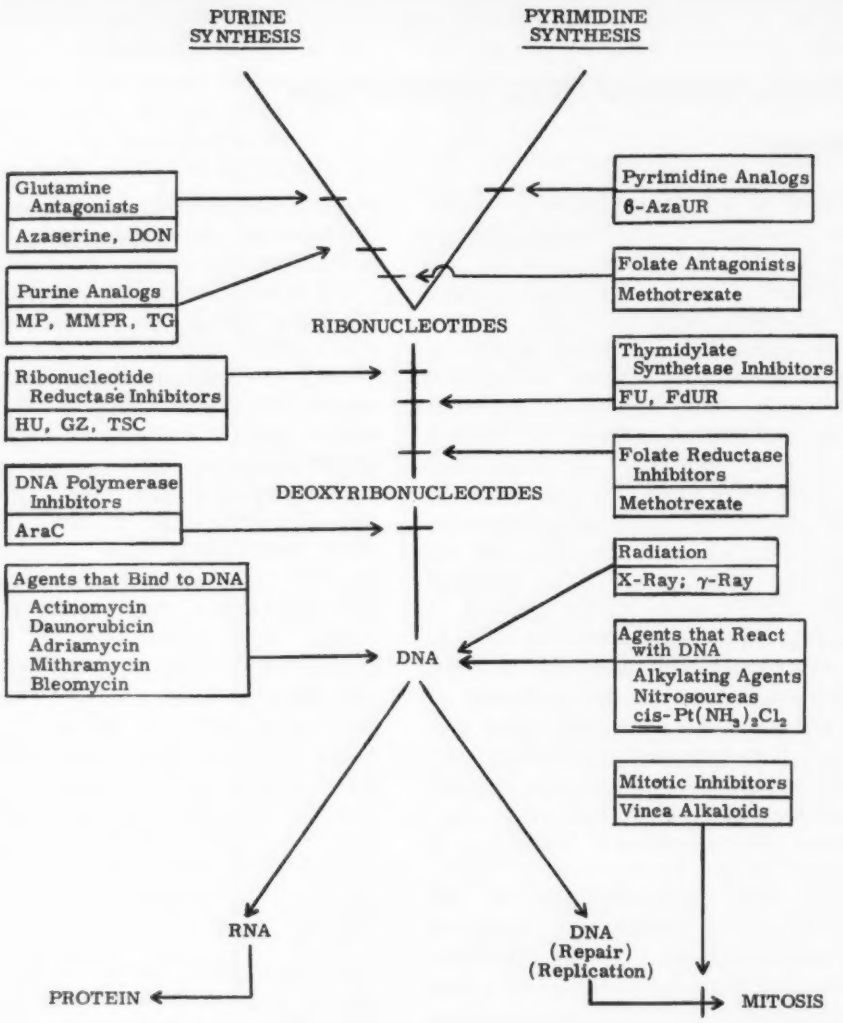


FIGURE 1.—Loci of action of anticancer agents on the synthesis of macromolecules and on cell division. Abbreviations: DON = 6-diazo-5-oxo-L-norleucine; MP = 6-mercaptopurine; MMPR = 6-methylmercaptopurine ribonucleoside; TG = 6-thioguanine; HU = hydroxyurea; GZ = guanazole; TSC = α-(*N*)-heterocyclic aldehyde thiosemicarbazones; AraC = arabinosylcytosine; 6-AzaUR = 6-azauridine; FU = 5-fluorouracil; FdUR = 5-fluoro-2′-deoxyuridine; *Cis*-$Pt(NH_3)_2Cl_2$ = *cis*-diamminedichloroplatinum.

INHIBITION OF ENZYMIC DEGRADATION OF ANTICANCER AGENTS

One means by which the effectiveness of an agent might be enhanced is to block the degradation of the administered drug (fig 2). For example, the degradation of 8-azaguanine by guanine aminohydrolase is effectively inhibited by 4-amino-5-imidazolecarboxamide (30); 9-(*p*-bromoacetamidophenyl)guanine was also reported to inhibit this enzyme activity (35). The oxidation of 6-thiopurine to 6-thioxanthine can be blocked by 4-hydroxypyrazolo-(3,4-*d*)pyrimidine, an inhibitor of xanthine oxidase (36). The enzymic deamination of ara-C to arabinosyluracil, an inactive metabolite, is inhibited by tetrahydrouridine (37). In

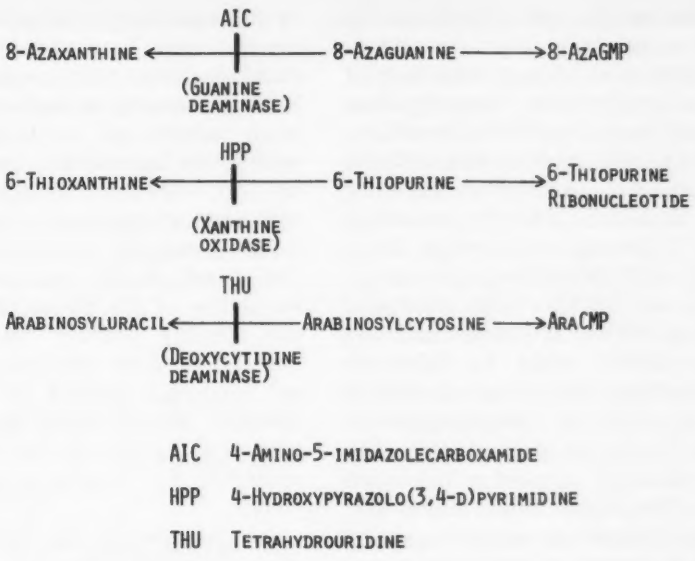


FIGURE 2.—Inhibition of degradation of anticancer agents.

each of the above examples the compound that acts to inhibit degradation of the potential anticancer agent is itself without anticancer activity. Combinations such as these are one means by which the concentration of an inhibitor might be maintained in vivo, but unless such combinations offer a basis for selective effects on the neoplastic cell they are likely to result in an increased host toxicity of a given dose of drug.

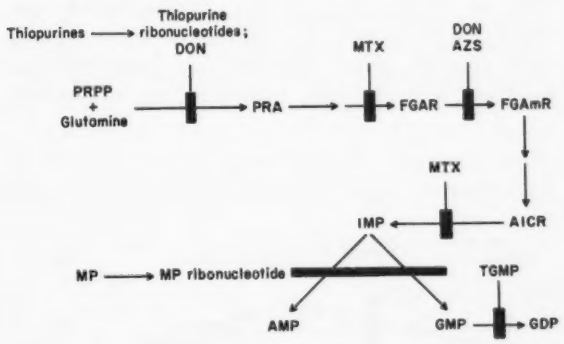


FIGURE 3.—Inhibition of purine ribonucleotide biosynthesis by purine analogs, by glutamine antagonists, and by methotrexate. Abbreviations: PRPP = 5-phosphoribosyl-1-pyrophosphate; PRA = 5-phosphoribosylamine; FGAR = formylglycinamide ribonucleotide; FGAmR = formylglycinamide ribonucleotide; AICR = 4-amino-5-imidazolecarboxamide; MTX = methotrexate; AZS = azaserine; TGMP = 6-thioguanylic acid. (*See* legend to fig 1 for abbreviations of other inhibitors.)

COMBINATIONS OF AGENTS THAT BLOCK SEQUENTIAL STEPS IN A PATHWAY

Some years ago, Dr. Van Potter put forth the hypothesis that agents that blocked sequential steps in a pathway might be used effectively in combination (38). Webb (39) and Handschumacher (40) have re-examined this concept from the standpoint of enzyme kinetics and have expressed reservations about its validity. Nevertheless, the hypothesis has stimulated experimental examination of drug combinations (41), some of which will be discussed.

Figure 3 summarizes the loci of action of three different classes of agents that affect purine biosynthesis. Thiopurine ribonucleotides (42,43) and 6-diazo-5-oxo-L-norleucine (DON) (44) block the synthesis of 5-phosphoribosylamine, an early step on the purine biosynthetic pathway. As indicated in figure 3, methotrexate blocks the biosynthesis of inosinic acid at two loci (4). The glutamine antagonists DON and azaserine inhibit the synthesis of formylglycinamide ribonucleotide (44). 6-Mer-

captopurine ribonucleotide can inhibit the interconversion of purine ribonucleotides (45) in addition to the already mentioned inhibition of phosphoribosylamine synthesis. Opportunities for sequential inhibition of purine biosynthesis are evident, and various combinations of these agents have been examined. For example, Stock and his associates (46,47) examined combinations of glutamine antagonists (azaserine and DON) with thiopurines (6-mercaptopurine [6-MP] and 6-TG) and concluded that synergistic inhibition of growth of some experimental neoplasms could be achieved. Sartorelli and LePage (48) reached similar conclusions in a study of combinations of azaserine and 6-TG. Goldin and Mantel (49) noted that low doses of azaserine increased the effectiveness of low doses of 6-MP or 6-TG; however, the same therapeutic results could be obtained with higher doses of the purine analogs alone; the importance of the scheduling of agents in combination was recognized. Combinations of purine analogs and glutamine antagonists have not found use in the treatment of cancer.

The combination of methotrexate and 6-MP was examined by Skipper and his colleagues (50,51) and by Law et al (52); these investigators concluded that the combination of agents was more effective than either agent alone against the parent sensitive L1210 leukemia. Goldin and Mantel (49) and Venditti et al (53), by their analysis of the effectiveness of the combination of methotrexate plus 6-MP against early and advanced L1210 leukemia, concluded that this combination did not exhibit therapeutic synergism. However, a significant aspect of combination chemotherapy with these agents was recognized by Law et al (52), who observed increased sensitivity to methotrexate in mouse leukemias resistant to 6-MP. Thus, the development of resistance to one agent, 6-MP, resulted in increased vulnerability of the resistant cells to methotrexate offering another basis for the possible efficacy of this combination and illustrating an important concept in combination chemotherapy. Methotrexate and 6-MP in combination with other agents are used in clinical protocols for treatment of acute leukemias (54).

An interesting and instructive example of chemotherapy with agents that exert inhibitory effects at multiple loci on the purine biosynthetic pathway is afforded by the combination of 6-methylmercaptopurine ribonucleoside (6-MMPR) and 6-MP or 6-TG (fig 4). Interest in 6-MMPR was renewed when it was observed in these laboratories that sublines of L1210 resistant to 6-MP were sensitive to treatment with 6-MMPR (55). Also, it was demonstrated that 6-MMPR was an inhibitor of the formation of formylglycinamide ribonucleotide in 6-MP-resistant cells in culture, in vitro and in vivo (55,56). More specifically, 6-methylthiopurine ribonucleotide, produced by intracellu-

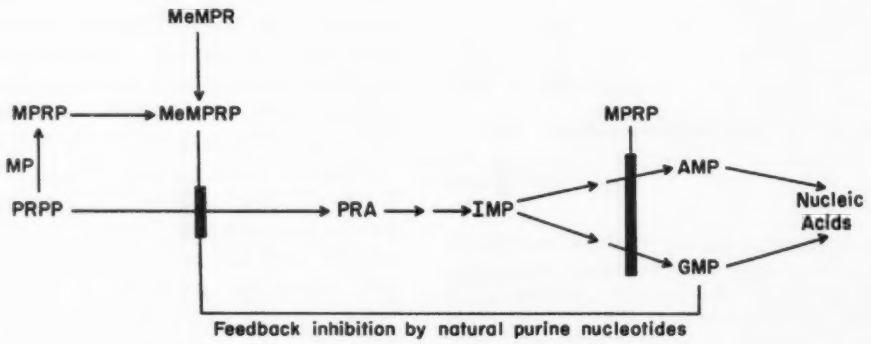
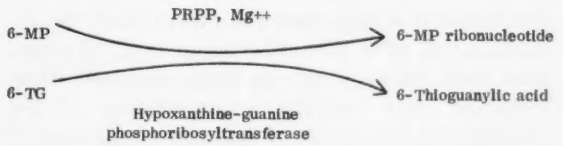


FIGURE 4.—Inhibition of purine synthesis de novo by 6-thiopurines. Abbreviations: MPRP = 6-mercaptopurine ribonucleotide; MeMPR = 6-methylmercaptopurine ribonucleoside; MeMPRP = 6-methylmercaptopurine ribonucleotide. (*See* legends to figs 1 and 2 for other abbreviations.)

lar phosphorylation of the ribonucleoside by adenosine kinase (57,58), was found to be a potent inhibitor of the synthesis of 5-phosphoribosylamine (43), the first step on the purine biosynthetic pathway. A consequence of such inhibition is an elevation of the level of 5-phosphoribosyl-1-pyrophosphate (PRPP) in inhibited cells (59). The increased availability of PRPP results in enhanced enzymic synthesis of ribonucleotides from base analogs, such as 6-MP (59,60) and 6-TG (61).



The combination of 6-MP and 6-MMPR was demonstrated to be therapeutically potentiating against L1210 leukemia in vivo (62) and against Ehrlich ascites carcinoma in vivo (63). The combination produces a dual block on the purine biosynthetic pathway as depicted in figure 4. When 6-TG is used in place of 6-MP then 6-thioguanylic acid is formed, which may exert multiple blocks as shown in figure 5.

As discussed earlier, the incorporation of 6-TG into DNA probably is a major factor in the lethal effect of this agent. Treatment with 6-MMPR and 6-TG results in increased formation of 6-thioguanylic acid by the mechanism discussed above. It should be pointed out that a biochemical basis for *selective* effects of these combinations on tumor cells has not yet been adequately described; more rapid recovery of affected host tissue, such as bone marrow, than of neoplastic cells probably is a factor.

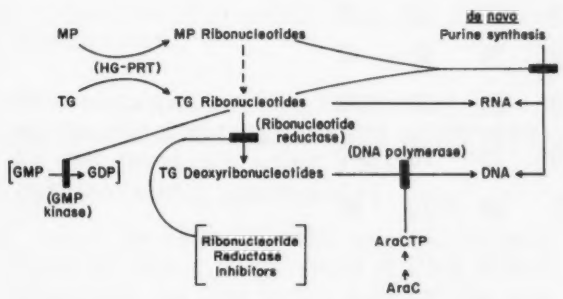


FIGURE 5.—Metabolism of 6-TG and loci of action of nucleotides of 6-TG. (*See* legends to previous figs for abbreviations.)

Combinations of 6-TG with inhibitors of DNA synthesis, such as ara-C and certain inhibitors of ribonucleotide reductase, have proven to be of interest in experimental chemotherapy. Such combinations may be classed as examples of complementary inhibitors.[3] The origin of the combination of ara-C and 6-TG stems from earlier work of Burchenal and Dollinger (65), who observed in L1210 mouse leukemia that combinations of ara-C and 6-MP produced less-than-additive toxic effects with improved therapeutic effectiveness. Gee et al (66) and Clarkson et al (67) treated adult acute leukemia with combinations of ara-C plus 6-TG with encouraging results. Schmidt and associates (68) then made an extensive evaluation of this combination in experimental animals and demonstrated that simultaneous administration of the two drugs resulted in increased therapeutic effectiveness of the combination with concomitant decrease in toxicity of 6-TG. That is, the toxicity of the two drugs was less than additive. A number of drug combinations, including ara-C plus 6-TG, were evaluated in a study of the toxic effects of drug combinations. A combination toxicity index was devised (69) based on determinations of LD10 doses in mice for individual agents and on the sum of decimal fractions of the individual LD10 doses for the combination. By the criteria used, a combination toxicity index of 1.0 indicates additive toxicity of the two agents; values greater than 1.0 indicate less-than-additive toxicity; values less than 1.0 indicate greater-than-additive toxicity. The toxicity indices for 6-TG in combination with ara-C or with ribonucleotide reductase inhibitors are summarized in table 1. The ara-C plus 6-TG index of 2.3 is quite favorable, and therapeutic potentiation of this combination in the treatment of animals implanted with 10^6 L1210 cells intravenously (iv) was convincingly demonstrated (*see* ref 69, table 3). Guanazole, an inhibitor of the synthesis of deoxyribonucleo-

[3] Sartorelli (64) describes complementary inhibitors as agents that cause damage to macromolecules by altering their structure either directly or indirectly in combination with drugs that decrease the availability of precursors of macromolecules.

TABLE 1.—Combination toxicity index for 6-TG and inhibitors of DNA synthesis

COMBINATION			INDEX
ARABINOSYLCYTOSINE	(ARAC)	+ TG	2.3
HYDROXYUREA	(HU)	+ TG	1.6
GUANAZOLE	(GZ)	+ TG	> 2.0
THIOSEMICARBAZONE	(HPTSC)	+ TG	1.1

TOXICITY INDEX: ADDITIVE (1.0); LESS THAN ADDITIVE (>1.0); GREATER THAN ADDITIVE (<1.0) (SEE REF. 70)

tides (8), in combination with 6-TG also gave a favorable toxicity index (table 1), and this encouraged us to evaluate this combination for therapeutic potentiation. Intraperitoneal (ip) treatment of mice bearing 10^5 L1210 cells with 6-TG alone did not result in long-term survivors; guanazole alone at high doses was effective (table 2). Combinations of low doses of guanazole with a range of doses of 6-TG resulted in a significantly increased survival rate. Also, it was possible to administer higher doses of 6-TG in the presence of guanazole without increased toxicity. Although not as active as ara-C plus 6-TG, the guanazole plus 6-TG combination is of interest as another example of complementary inhibition. Since guanazole alone gave favorable responses in the treatment of adult acute leukemia (70), the combination of guanazole with 6-TG merits consideration.

A basis for the decreased toxicity of 6-TG in the presence of inhibitors of DNA synthesis may be inferred from figure 5. Ara-C and ribonucleotide reductase inhibitors block the incorporation of 6-TG into nucleic acids of sensitive host tissue, such as bone marrow, thus reducing host toxicity. Incorporation of the analog into the DNA of leukemic cells also is inhibited by these agents. Therefore, if incorporation of 6-TG into DNA is primarily responsible for its contribution to the antileukemic activity of the combination, then it becomes necessary to postulate differential recovery of the tumor and host cells from inhibition of DNA synthesis in such a way that tumor cells are subjected to lethal effects of incorporation of the analog. An alternate pos-

TABLE 2.—Combination chemotherapy for L1210 leukemia with 6-TG plus guanazole

IMPLANT: 10^5 L1210 CELLS IP
TREATMENT SCHEDULE: Q3HR X 8; DAYS 1,5,9; IP

60-DAY SURVIVORS (%)

GUANAZOLE (LD_{10} 540) (MG/KG)	6-THIOGUANINE (LD_{10} 0.54) 0	0.16	0.24	0.36	0.54	0.81
0		0	0	0	0	0
162	0	22	0	30	30	70
238	0	0	0	10	50	100
362	20	30	20	70	70	100
540	70	70	90	80	70	80
810	30					

CONDENSED SUMMARY OF RESULTS (DR. F.M.SCHABEL,JR., DR. W.R. LASTER, JR., AND MRS. MARY TRADER).

COMBINATION TOXICITY INDEX = 2.0 OR >.

TABLE 3.—Effect of deferoxamine on ribonucleotide reductase of H.Ep.-2 cells

Reaction Mixture	Ribonucleotide reductase activity: Nanomoles dCyR per mg Protein per 30 min	Ribonucleotide reductase activity: % of Control
Complete	14.1	100
-Mg^{++}	17.1	121
-Fe^{++}	10.0	71
-Mg^{++}; -Fe^{++}	5.4	38
Complete	8.4	100
+ Deferoxamine		
6×10^{-5} M	6.2	74
7.2×10^{-5} M	0.8	10
1.2×10^{-4} M	0.6	7
+ Deferoxamine + Fe^{++}		
1.2×10^{-4} M + 1.2×10^{-4} M	8.4	100

THE COMPLETE REACTION MIXTURE FOR REDUCTION OF CDP-2-^{14}C IS THAT OF E.C. MOORE (82) AND CONTAINS Mg^{++} (2.7×10^{-3} M) AND Fe^{++} (6×10^{-5} M).

tulate is that inhibitors of DNA synthesis prevent incorporation of 6-TG into the DNA of sensitive cells but do not block this process in cells resistant to ara-C or resistant to ribonucleotide reductase inhibitors. Schabel (71) and Skipper (69) observed that ara-C alone fails in the treatment of mice implanted with 10^7 leukemia cells, probably as a consequence of the overgrowth of a drug-resistant population. Gailani and Nussbaum (72) also have observed the development of resistance to ara-C as a consequence of metabolic failure to phosphorylate ara-C in human leukemia cells. Although experimental chemotherapy of leukemia with 6-TG alone is not particularly effective, the drug may serve an effective role in the combination by elimination of ara-C-resistant cells that arise during the course of treatment (73).

ENHANCEMENT OF THE EFFECT OF RIBONUCLEOTIDE REDUCTASE INHIBITORS BY THE IRON-CHELATING AGENT DEFEROXAMINE (DESFERAL®)

The enzyme ribonucleotide reductase occupies a key position in the synthesis of DNA in that it provides the only known pathway for conversion of ribonucleotides synthesized de novo to deoxyribonucleotides, the immediate pre-

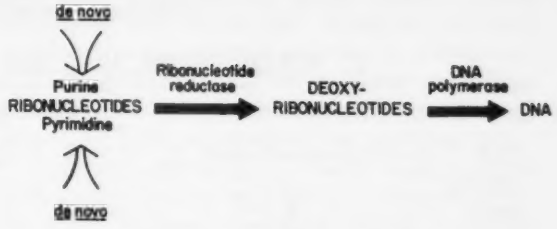


FIGURE 6.—Key role of ribonucleotide reductase in the synthesis of DNA.

cursors of DNA (fig 6). Several structurally different agents are known to inhibit this enzyme: hydroxyurea (74–76), guanazole (8), and certain heteroaromatic aldehyde thiosemicarbazones (9–11,77). The latter class of compounds was recognized as having antileukemic activity some years ago (78) and was further developed by French and his associates (79,80). Also, certain of these thiosemicarbazones are the most potent inhibitors of ribonucleotide reductase known.

Iron (Fe^{++}) is required for the enzymatic reduction of ribonucleotides; chelation of iron by deferoxamine inhibits the enzymatic reduction of ribonucleotides and such inhibition can be alleviated by iron salts (table 3). We observed that inhibition of growth and inhibition of DNA synthesis produced in tumor cells in culture by inhibitors of ribonucleotide reductase could be enhanced by the presence of deferoxamine (82). Laster (83) observed that deferoxamine, which is itself devoid of antileukemic activity, enhanced the activity of 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone against 10^6 ip implanted L1210 cells (table 4). At LD10 or less doses of the combination there were significant numbers of long-term survivors, whereas either drug alone was ineffective. Also, the combination therapy resulted in significant increases in lifespan of dying animals over that observed with individual agents. It is possible that lower doses of a thiosemicarbazone could be used in combination with deferoxamine as a means of moderating toxicity encountered in the clinical use of thiosemicarbazones (84,85). The α-(*N*)-heterocyclic aldehyde thiosemicarbazones are themselves capable of chelating iron (9,10,77,-

TABLE 4.—Combination treatment of L1210 leukemia with deferoxamine (Desferal) and 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone (5-H-P2-CATSC)

1. PERCENT SURVIVAL AT 60 DAYS OF NONTUMOR-BEARING BDF_1 MICE.

DRUG (MG/KG/DOSE) 5-H-P2-CATSC ↓ / DESFERAL →	0	83	125	188	280	420	
0		-	-	100	100	100	
30	100	-	-	100	100	100	ALL DOSES ABOVE THIS
44	90	100	90	100	100	80	← LINE ≤ LD_{10}
66	30	-	80	100			

2. 60TH-DAY SURVIVORS (%) OF LEUKEMIC BDF_1 MICE (10^6 CELLS).

DRUG (MG/KG/DOSE) 5-H-P2-CATSC ↓ / →	0	83	125	188	280	420	
0		-	-	0	0	0	
30	0	-	-	50	30	30	ALL DOSES ABOVE THIS
44	0	30	(50)	40	30	40	← LINE ≤ LD_{10}
66	0	-	30	44			

3. PERCENT I.L.S. OF LEUKEMIC BDF_1 MICE BASED ON MEDIAN DAY OF DEATH (SURVIVORS NOT INCLUDED).

DRUG (MG/KG/DOSE) 5-H-P2-CATSC ↓ / →	0	83	125	188	280	420	
0				-13	-13	-7	
30	(+106)	-	-	+125	+125	+150	ALL DOSES ABOVE THIS
44	+93	+150	(+237)	+175	+175	+262	← LINE ≤ LD_{10}
66	+31	-	+137	+175			

79,80,86,87); the use of deferoxamine would not be indicated if toxic manifestations of thiosemicarbazones are related to reduced iron levels.

COMBINATION OF PHASE-SPECIFIC AND NON-PHASE-SPECIFIC AGENTS

Bases for combinations, such as the examples given in figure 7, can be drawn from consideration of cell kinetics and also from the observation that resistance develops readily in large populations of tumor cells treated with ara-C alone (69,71). The use of ara-C in combination with alkylating agents, such as the nitrosoureas (which have the capability of killing cells that are not in cycle), resulted in dramatic improvement in the therapy of mouse leukemia (71).

Therapy with ara-C, an S-phase-specific agent, produces some degree of synchrony within a tumor cell population. It has been reported that cells in the G_1/S "boundary" phase and cells in mid-S phase are somewhat more sensitive to alkylating agents than are cells in other phases of the cell cycle (88). These are factors which probably contribute to the efficacy of combinations of ara-C and alkylating agents.

COMBINATION OF DNA-BINDING AGENTS WITH ALKYLATING AGENTS

Schabel (89) discussed experimental chemotherapy with drugs that bind to DNA in combination with other anticancer agents, some of which are known to react with DNA or to yield intermediates which react with DNA. Some combinations are suggested in figure 8. The combination of adriamycin and DIC (NSC-45388) is of particular interest in view of the observation by Gottlieb et al (90) on the effectiveness of this combination in the treatment of metastatic sarcomas in man.

A possible basis for the effectiveness of com-

PHASE-SPECIFIC		NON-PHASE-SPECIFIC
ARAC	+	CYCLOPHOSPHAMIDE BCNU CCNU

POSTULATES:

1. ALKYLATING AGENTS AND NITROSOUREAS KILL ARAC-RESISTANT CELLS AS WELL AS PARENT CELLS IN THE TUMOR CELL POPULATION.

2. ARAC TREATMENT RESULTS IN SOME SYNCHRONY WITHIN THE TUMOR CELL POPULATION; ALKYLATING AGENTS ARE SOMEWHAT MORE EFFECTIVE AGAINST CELLS IN G_1/S "BOUNDARY" AND MID-S.

3. TREATMENT WITH NON-PHASE-SPECIFIC AGENTS RESULTS IN AN INCREASED GROWTH FRACTION WITH CONSEQUENT INCREASED SENSITIVITY TO ARAC.

FIGURE 7.—Combination of phase-specific and non-phase-specific agents.

binations such as those suggested in figure 8 is that the repair of DNA damaged by alkylating agents may be hampered by the presence of the agent that is bound to DNA, such as adriamycin, daunorubicin, or mithramycin.

COMBINATIONS OF AGENTS THAT INHIBIT DNA SYNTHESIS AND AGENTS THAT PRODUCE BREAKS IN DNA

Ara-C is well known as a specific inhibitor of DNA synthesis. Bleomycin produces scission and fragmentation of DNA (91,92) with release of free bases (93). Exposure of cells in culture to camptothecin results in fragmentation of cellular DNA to a lower molecular weight DNA (94); how this relates to observed effects on RNA synthesis is not yet clear (95).

Combinations of ara-C with bleomycin or with camptothecin (fig 9) are suggested on the basis that ara-C would inhibit synthesis of new DNA, while treatment with bleomycin or camptothecin would result in degradation of existing cellular DNA. Such combinations bear an obvious analogy to the combination of actinomycin D and ribonuclease, which resulted in synergistic inhibition of growth of Sarcoma 180 with accompanying depletion of cellular RNA (96).

DNA BINDER		ALKYLATING AGENT
ADRIAMYCIN	+	MECCNU BIC DIC

POSTULATES:

1. ALKYLATION OF DNA; INHIBITION OF DNA REPAIR BY THE AGENT BOUND TO DNA.

2. ACTION OF ALKYLATING AGENTS AND DNA BINDERS MAY BE INDEPENDENT BUT SUPPLEMENTARY IN EFFECTS ON REPLICATION OR REPAIR OF DNA.

FIGURE 8.—Combination of DNA binding agents with alkylating agents.

COMBINATIONS OF ALKYLATING AGENTS WITH POTENTIAL INHIBITORS OF REPAIR

Valeriote et al (97) obtained evidence for therapeutic potentiation by the use of alkylating agents in combination. These investigators examined cyclophosphamide in combination with BCNU against transplanted AKR lymphoma. Schabel (98) confirmed this observation and extended it to L1210 leukemia. More recently, Mayo et al (99) showed that the combination of cyclophosphamide with methyl-CCNU gave therapeutic potentiation against Lewis lung carcinoma. Combinations of nitrosoureas with certain triazenes were examined for therapeutic response against L1210 leukemia, and it was observed that a combination of CCNU with BIC (NSC-82196), a compound capable of generating an alkylating agent (100–102), proved to be particularly interest-

INHIBITORS OF DNA SYNTHESIS		AGENTS THAT FRAGMENT DNA
ARAC	+	BLEOMYCIN CAMPTOTHECIN

POSTULATE:

ARAC INHIBITS THE SYNTHESIS OF DNA AND BLEOMYCIN OR CAMPTOTHECIN MAY CAUSE DEGRADATION OF DNA.

FIGURE 9.—Combination of agents that inhibit DNA synthesis with agents that produce breaks in DNA.

Alkylating Agent		Inhibitor of Repair
BIC DIC	+	CCNU
Cyclophosphamide	+	MeCCNU

POSTULATE:

Alkylation of DNA; inhibition of repair by nitrosoureas as a result of carbamoylation of repair enzymes.

Figure 10.—Combinations of alkylating agents with potential inhibitors of repair.

ing; therapeutic potentiation was observed in mice implanted iv with 10^7 L1210 cells (89).

The examples given in figure 10 of combinations of two agents, each of which has potential alkylating activity, pose an interesting challenge to those of us who attempt to place biochemical interpretations on these chemotherapy data as part of an effort to contribute to rational approaches to cancer chemotherapy. The nitrosoureas have the capacity to form highly reactive carbonium ions, which can alkylate nucleic acids; nitrosoureas also have the capacity to form isocyanates, which can react with the amino groups of amino acids and proteins (103) (*see* fig 11). It was postulated by Dr. G.P. Wheeler (personal communication) that nitrosoureas might thus have the dual capacity of alkylating DNA and, by carbamoylation of protein, of inactivating enzymes necessary for repair of damaged DNA. This postulate is applied here to suggest that substituted triazenoimidazole carboxamides alkylate DNA and that nitrosoureas, in addition to alkylation of DNA, may also inhibit repair of damaged DNA. A similar interpretation can be placed on combinations of cyclophosphamide with nitrosoureas.

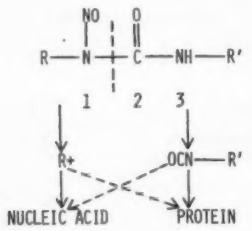


Figure 11.—Generalized structure of 1,3-disubstituted nitrosoureas, the biologically active products derived by their decomposition, and probable loci of biologic activity. The nitrosoureas can break down chemically to yield a carbonium ion, designated R^+, and an isocyanate, designated R'–NCO. The primary action of the carbonium ion is considered to be alkylation of DNA; the primary action of the isocyanate moiety probably is reaction with amino groups of protein.

COMMENTS

From what has gone before, the reader may note that in the realm of combination chemotherapy the biologic observation frequently precedes the efforts of the biochemist to understand the basis for the efficacy of such therapeutic regimens. This situation is reminiscent of the physical chemist who, after much study, informs the organic chemist that the chemical reaction by which he has successfully accomplished the synthesis of a new compound is thermodynamically possible. Although the activities of many antitumor agents and of combinations of agents have been discovered empirically, biochemical information has contributed to the development of new agents, of new therapeutic regimens, and of new combinations of agents. As knowledge of mechanisms of action of anticancer agents, both old and new, increases, the possibilities for developing rational bases for combinations of agents also increase.

It is important also to understand biochemical mechanisms by which neoplasms become resistant to agents that inhibit their growth. The biochemist may be able to offer useful advice to his colleague in chemotherapy as to potential combinations based on knowledge of resistance mechanisms. Also, there are interesting examples of increased sensitivity to a chemotherapeutic agent of neoplastic cells resistant to a different agent. The example of increased sensitivity to methotrexate of cells resistant to purine analogs was mentioned. Recently we described the increased sensitivity of ara-C-resistant L1210 cells to 3-deazauridine (104), providing another example of

the interesting phenomenon of collateral sensitivity (105,106).

There is every reason to think that there will continue to be many opportunities for biochemical contributions to the chemotherapy of cancer, a subject that is scarcely 25 years old. Attempts at rational combination chemotherapy sometimes lead us " . . . to do the right deed for the wrong reason,"[4] but this should not deter us from the effort.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127

Arabinosylcytosine: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytosine arabinoside; ara-C

8-Azaguanine: NSC-749; CAS reg. No. 529-99-7; 7*H*-*v*-triazolo[4,5-*d*]pyrimidin-7-one, 5-amino-1,6-dihydro-

Azaserine: NSC-742; CAS reg. No. 115-02-6; serine, diazoacetate (ester), L-

6-Azauridine: NSC-32074; *as*-triazine-3,5(2*H*,4*H*)-dione, 2-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

***Cis*-diamminedichloroplatinum:** NSC-119875; CAS reg. No. 15663-27-1

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

3-Deazauridine: NSC-126849; 2,4(1*H*,3*H*)-pyridinedione, 1-β-D-ribofuranosyl-

6-Diazo-5-oxo-L-norleucine: NSC-7365; DON

5-Fluoro-2'-deoxyuridine: NSC-27640; 5-FUDR

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Guanazole: NSC-1895; CAS reg. No. 503-88-8; 1,2,4-triazolidine, 3,5-diimino-

5-Hydroxypyridine-2-carboxaldehyde thiosemicarbazone: NSC-107392; CAS reg. No. 19494-89-4; 5-hydroxypicolinaldehyde thiosemicarbazone

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; amethopterin

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

6-Methylmercaptopurine ribonucleoside: NSC-40774; CAS reg. No. 7143-67-1; 9*H*-purine, 6-(methylthio)9-β-D-ribofuranosyl-, dihydrate

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; DIC

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-; BIC

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

[4] Adapted from "Murder in the Cathedral," by T.S. Eliot.

REFERENCES

1. LIVINGSTON RB, VENDITTI JM, COONEY DA, ET AL. Glutamine antagonists in chemotherapy. *In* Advances in Pharmacology and Chemotherapy (Garattini S, Goldin A, Hawking F, et al, eds). New York, Academic Press, 1970, vol 8, pp 57–120.
2. HITCHINGS GH, and ELION GB. Purine analogues. *In* Metabolic Inhibitors (Hochster RM, and Quastel JH, eds). New York, Academic Press, 1963, vol 1, pp 215–237.
3. BROCKMAN RW. Mechanisms of resistance to anticancer agents. *In* Advances in Cancer Research (Weinhouse S, and Haddow A, eds). New York, Academic Press, 1963, vol 7, pp 129–234.
4. BLAKLEY RL. The biochemistry of folic acid and related pteridines. *In* Frontiers of Biology. Amsterdam, North Holland Publishing Co., 1969, vol 13.
5. HANDSCHUMACHER RE, and WELCH AD. Agents which influence nucleic acid metabolism. *In* The Nucleic Acids (Chargaff E, and Davidson JN, eds). New York, Academic Press, 1960, vol 3, pp 453–526.
6. ELFORD HL. Effect of hydroxyurea on ribonucleotide reductase. Biochem Biophys Res Commun 33:129–135, 1968.
7. KRAKOFF IH, BROWN NC, and REICHARD P. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res 28:1559–1565, 1968.
8. BROCKMAN RW, SHADDIX S, LASTER WR, JR, ET AL. Inhibition of ribonucleotide reductase, DNA synthesis, and L1210 leukemia by guanazole. Cancer Res 30:2358–2368, 1970.
9. MOORE EC, ZEDECK MS, AGRAWAL KC, ET AL. Inhibition of ribonucleoside diphosphate reductase by 1-formylisoquinoline thiosemicarbazone and related compounds. Biochemistry 9:4492–4498, 1970.
10. MOORE EC, BOOTH BA, and SARTORELLI AC. Inhibition of deoxyribonucleotide synthesis by pyridine carboxaldehyde thiosemicarbazones. Cancer Res 31:235–238, 1971.
11. BROCKMAN RW, SIDWELL RW, ARNETT G, ET AL. Heterocyclic thiosemicarbazones: correlation between structure, inhibition of ribonucleotide reductase, and inhibition of DNA viruses. Proc Soc Exp Biol Med 133:609–614, 1970.

12. HEIDELBERGER C. Fluorinated pyrimidines. *In* Progress in Nucleic Acid Research and Molecular Biology (Davidson JN, and Cohn WE, eds). New York, Academic Press, 1965, vol 4, pp 1–50.
13. FURTH JJ, and COHEN SS. Inhibition of mammalian DNA polymerase by the 5'-triphosphate of 1-β-D-arabinofuranosylcytosine and the 5'-triphosphate of 9-β-D-arabinofuranosyladenine. Cancer Res 28:2061–2067, 1968.
14. KIMBALL AP, and WILSON MJ. Inhibition of DNA polymerase by β-D-arabinosylcytosine and reversal of inhibition by deoxycytidine-5'-triphosphate. Proc Soc Exp Biol Med 127:429–432, 1968.
15. ELKIND MM, and WHITMORE GF. The Radiobiology of Cultured Mammalian Cells. New York, Gordon & Breach, 1967.
16. ALTMAN KI, GERBER GB, and OKADA S. Radiation Biochemistry. New York, Academic Press, 1970, vol 1.
17. WHEELER GP. Some biochemical effects of alkylating agents. Fed Proc 26:885–890, 1967.
18. LAWLEY PD. Effects of some chemical mutagens and carcinogens on nucleic acids. *In* Progress in Nucleic Acid Research and Molecular Biology (Davidson JN, and Cohn WE, eds). New York, Academic Press, 1966, vol 5, pp 89–131.
19. NEWTON BA. Chemotherapeutic compounds affecting DNA structure and function. *In* Advances in Pharmacology and Chemotherapy (Garattini S, Goldin A, Hawking F, et al, eds). New York, Academic Press, 1970, vol 8, pp 149–184.
20. CHENG CJ, FUJIMURA S, GRUNBERGER D, ET AL. Interaction of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037) with nucleic acids and proteins in vivo and in vitro. Cancer Res 32:22–27, 1972.
21. CARTER SK, SCHABEL FM, JR, BRODER LE, ET AL. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and other nitrosoureas in cancer treatment: a review. *In* Advances in Cancer Research (Klein G, Weinhouse S, and Haddow A, eds). New York, Academic Press, 1972, vol 16, pp 273–332.
22. HOWLE JA, GALE GR, and SMITH AB. A proposed mode of action of antitumor platinum compounds based upon studies with *cis*-dichloro ([G-^{3}H]-dipyridine)platinum (II). Biochem Pharmacol 21:1465–1475, 1972.
23. SZYBALSKI W, and IYER VN. The mitomycins and porfiromycins. *In* Antibiotics. I: Mechanisms of Action (Gottlieb D, and Shaw PD, eds). New York, Springer-Verlag, 1967, pp 211–245.
24. HARTMANN G, BEHR W, BEISSNER K-A, ET AL. Antibiotics as inhibitors of nucleic acid and protein synthesis. Angew Chem 7:693–701, 1968.
25. UMEZAWA H, MAEDA K, TAKEUCHI T, ET AL. New antibiotics, bleomycin A and B. J Antibiot 19:200–209, 1966.
26. SUZUKI H, NAGAI K, AKUTSU E, ET AL. On the mechanism of action of bleomycin. Strand scission of DNA caused by bleomycin and its binding to DNA in vitro. J Antibiot 23:473–480, 1970.
27. TERASIMA T, YASUKAWA M, and UMEZAWA H. Breaks and rejoining of DNA in cultured mammalian cells treated with bleomycin. Gann 61:513–516, 1970.
28. MÜLLER WEG, YAMAZAKI Z, and ZAHN RK. Bleomycin, a selective inhibitor of DNA-dependent DNA polymerase from oncogenic RNA viruses. Biochem Biophys Res Commun 46:1667–1673, 1972.
29. CREASEY WA, BENSCH KG, and MALAWISTA SE. Colchicine, vinblastine and griseofulvin. Pharmacological studies with human leukocytes. Biochem Pharmacol 20:1579–1588, 1971.
30. MANDEL HG. The physiological disposition of some anticancer agents. Pharmacol Rev 11:743–838, 1959.
31. LEPAGE GA. Basic biochemical effects and mechanism of action of 6-thioguanine. Cancer Res 23:1202–1206, 1963.
32. BERGQUIST PL, and MATTHEWS REF. Effects of 8-azaguanine on the composition of ribonucleic acids from subcellular fractions. Biochem J 85: 313–319, 1962.
33. MANDEL HG. Comparative actions of some purine analogues on growing *Bacillus cereus*. Fed Proc 26:905–906, 1967.
34. MANDEL HG. The incorporation of fluorouracil into RNA and its molecular consequences. *In* Progress in Molecular and Subcellular Biology. New York, Springer, 1969, vol 1, pp 82–135.
35. BAKER BR, and WOOD WF. Irreversible enzyme inhibitors. CIII. 9-(*p*-bromoacetamidophenyl)-guanine, an irreversible inhibitor of xanthine oxidase. J Med Chem 10:1106–1109, 1967.
36. FEIGELSON P, DAVIDSON JD, and ROBINS RK. Pyrazolopyrimidines as inhibitors and substrates of xanthine oxidase. J Biol Chem 226:993–1000, 1957.
37. CAMIENER GW. Studies of the enzymatic deamination of aracytidine. V. Inhibition in vitro and in vivo by tetrahydrouridine and other reduced pyrimidine nucleosides. Biochem Pharmacol 17: 1981–1991, 1968.
38. POTTER VR. Sequential blocking of metabolic pathways in vivo. Proc Soc Exp Biol Med 76:41–46, 1951.
39. WEBB JL. Enzyme and Metabolic Inhibitors. New York, Academic Press, 1963, vol 1, p 498.
40. HANDSCHUMACHER RE. Formal discussion: some enzymatic considerations in combination chemotherapy. Cancer Res 25: 1541–1543, 1965.
41. SARTORELLI AC. Approaches to combination chemotherapy of transplantable neoplasms. *In* Progress in Experimental Tumor Research (Homburger F, ed). New York, Hafner Publishing Co, 1965, vol 6, pp 228–288.
42. MCCOLLISTER RJ, GILBERT WR, JR, ASHTON DM,

ET AL. Pseudofeedback inhibition of purine synthesis by 6-mercaptopurine ribonucleotide and other purine analogues. J Biol Chem 239:1560–1563, 1964.

43. HILL DL, and BENNETT LL, JR. Purification and properties of 5-phosphoribosyl pyrophosphate amidotransferase from adenocarcinoma 755 cells. Biochemistry 8:122–130, 1969.
44. BUCHANAN JM, and HARTMAN SC. Enzymic reactions in the synthesis of the purines. *In* Advances in Enzymology (Nord FF, ed). New York, Interscience, 1959, vol 21, pp 199–261.
45. BALIS ME. Antagonists and nucleic acids. *In* Frontiers of Biology (Neuberger A, and Tatum EL, eds). Amsterdam, North Holland Publishing Co., 1968, vol 10.
46. CLARKE DA, REILLY HC, and STOCK CC. A comparative study of 6-diazo-5-oxo-L-norleucine and *O*-diazoacetyl-L-serine on sarcoma 180. Antibiot Chemother 7:653–671, 1957.
47. TARNOWSKI GS, and STOCK CC. Effects of combinations of azaserine and of 6-diazo-5-oxo-L-norleucine with purine analogs and other antimetabolites on the growth of two mouse mammary carcinomas. Cancer Res 17:1033–1039, 1957.
48. SARTORELLI AC, and LEPAGE GA. Inhibition of ascites cell growth by combinations of 6-thioguanine and azaserine. Cancer Res 18:938–942, 1958.
49. GOLDIN A, and MANTEL N. The employment of combinations of drugs in the chemotherapy of neoplasia: a review. Cancer Res 17:635–654, 1957.
50. SKIPPER HE. Effects of 6-mercaptopurine on experimental tumors. Ann NY Acad Sci 60:267–272, 1954.
51. SKIPPER HE, THOMSON JR, ELION GB, ET AL. Observations on the anticancer activity of 6-mercaptopurine. Cancer Res 14:294–298, 1954.
52. LAW LW, TAORMINA V, and BOYLE PJ. Response of acute lymphocytic leukemias to the purine antagonist 6-mercaptopurine. Ann NY Acad Sci 60:244–250, 1954.
53. VENDITTI JM, HUMPHREYS SR, MANTEL N, ET AL. Combined treatment of advanced leukemia (L1210) in mice with amethopterin and 6-mercaptopurine. J Natl Cancer Inst 17:631–638, 1956.
54. BODEY GP, and FREIREICH EJ. Acute leukemia. *In* Hematology: Principles and Practice (Mengel CE, Frei E III, and Nachman R, eds). Chicago, Year Book Medical Publishers, 1972, pp 381–423.
55. BENNETT LL, JR, BROCKMAN RW, SCHNEBLI HP, ET AL. Activity and mechanism of action of 6-methylthiopurine ribonucleoside in cancer cells resistant to 6-mercaptopurine. Nature 205: 1276–1279,1965.
56. HENDERSON JF, and KHOO MKY. On the mechanism of feedback inhibition of purine biosynthesis de novo in Ehrlich ascites tumor cells in vitro. J Biol Chem 240: 3104–3109, 1965.
57. BENNETT LL, JR, SCHNEBLI HP, VAIL MH, ET AL. Purine ribonucleoside kinase activity and resistance to some analogs of adenosine. Mol Pharmacol 2:432–443, 1966.
58. CALDWELL IC, HENDERSON JF, and PATERSON ARP. The enzymic formation of 6-(methylmercapto)purine ribonucleoside 5'-phosphate. Can J Biochem 44:229–245, 1966.
59. PATERSON ARP, and WANG MC. Mechanism of the growth inhibition potentiation arising from combination of 6-mercaptopurine with 6-(methylmercapto)purine ribonucleoside. Cancer Res 30:2379–2387, 1970.
60. SCHOLAR EM, BROWN PR, and PARKS RE, JR. Synergistic effect of 6-mercaptopurine and 6-methylmercaptopurine ribonucleoside on the levels of adenine nucleotides of Sarcoma 180 cells. Cancer Res 32:259–269, 1972.
61. NELSON JA, and PARKS RE, JR. Biochemical mechanisms for the synergism between 6-thioguanine and 6-(methylmercapto)purine ribonucleoside in sarcoma 180 cells. Cancer Res 32:2034–2041, 1972.
62. SCHABEL FM, JR, LASTER WR, JR, and SKIPPER HE. Chemotherapy of leukemia L1210 by 6-mercaptopurine (NSC-755) in combination with 6-methylthiopurine ribonucleoside (NSC-40774). Cancer Chemother Rep 51:111–124, 1967.
63. WANG MC, SIMPSON AI, and PATERSON ARP. Combinations of 6-mercaptopurine (NSC-755) and 6-(methylmercapto)purine ribonucleoside (NSC-40774) in therapy of Ehrlich ascites carcinoma. Cancer Chemother Rep 51:101–109, 1967.
64. SARTORELLI AC. Some approaches to the therapeutic exploitation of metabolic sites of vulnerability of neoplastic cells. Cancer Res 29:2292–2299, 1969.
65. BURCHENAL JH, and DOLLINGER MR. Cytosine arabinoside (NSC-63878) in combination with 6-mercaptopurine (NSC-755), methotrexate (NSC-740), or 5-fluorouracil (NSC-19893) in L1210 mouse leukemia. Cancer Chemother Rep 51:435–438, 1967.
66. GEE TS, YU K-P, and CLARKSON BD. Treatment of adult acute leukemia with arabinosylcytosine and thioguanine. Cancer 23:1019–1032, 1969.
67. CLARKSON BD, and FRIED J. Changing concepts of treatment in acute leukemia. Med Clin North Am 55:561–600, 1971.
68. SCHMIDT LH, MONTGOMERY JA, LASTER WR, JR, ET AL. Combination therapy with arabinosylcytosine and thioguanine. Proc Am Assoc Cancer Res 11:70, 1970.
69. SKIPPER HE. Combination therapy: some concepts and results. Cancer Chemother Rep Part 2, vol 4:137–145, 1974.
70. HEWLETT JS, BODEY GP, COLTMAN CA, ET AL. Intermittent guanazole therapy in adult acute leukemia. Clin Pharmacol Ther 14:271–276, 1973.
71. SCHABEL FM, JR. In vivo leukemic cell kill kinetics and "curability" in experimental systems. *In* The Proliferation and Spread of Neoplastic Cells

(21st Annual Symposium on Fundamental Cancer Research, M.D. Anderson Hospital and Tumor Institute). Baltimore, Williams and Wilkins, Co., 1967, pp 379–408.

72. GAILANI S, and NUSSBAUM A. Correlation of response to 1-β-D-arabinofuranosylcytosine (ara-C) and metabolism of drug by tumor. Proc Am Assoc Cancer Res 14:100, 1973.
73. LEPAGE GA, and WHITE SC. Scheduling of arabinosylcytosine and 6-thioguanine therapy. Cancer Res 33:946–949, 1973.
74. KRAKOFF IH, BROWN NC, and REICHARD P. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res 28:1559–1565, 1968.
75. ELFORD HL. Effect of hydroxyurea on ribonucleotide reductase. Biochem Biophys Res Commun 33:129–135, 1968.
76. MOORE EC. The effects of ferrous ion and dithioerythritol on inhibition by hydroxyurea of ribonucleotide reductase. Cancer Res 29:291–295, 1969.
77. SARTORELLI AC, AGRAWAL KC, and MOORE EC. Mechanism of inhibition of ribonucleoside diphosphate reductase by α-(*N*)-heterocyclic aldehyde thiosemicarbazones. Biochem Pharmacol 20:3119–3123, 1971.
78. BROCKMAN RW, THOMSON JR, BELL MJ, ET AL. Observations on the antileukemic activity of pyridine-2-carboxaldehyde thiosemicarbazone and thiocarbohydrazone. Cancer Res 16:167–170, 1956.
79. FRENCH FA, and BLANZ EJ, JR. The carcinostatic activity of thiosemicarbazones of formyl heteroaromatic compounds. 3. Primary correlation. J Med Chem 9:585–589, 1966.
80. BLANZ EJ, JR, and FRENCH FA. The carcinostatic activity of 5-hydroxy-2-formylpyridine thiosemicarbazone. Cancer Res 28:2419–2422, 1968.
81. MOORE EC. Mammalian ribonucleoside diphosphate reductases. *In* Methods in Enzymology (Colowick SP, and Kaplan NO, eds). New York, Academic Press, 1967, vol 12, pp 155–164.
82. BROCKMAN RW, SHADDIX S, STRINGER V, ET AL. Enhancement by deferoxamine of inhibition of DNA synthesis by ribonucleotide reductase inhibitors. Proc Am Assoc Cancer Res 13:88, 1972.
83. LASTER WR, JR, and BROCKMAN RW. Enhancement of the activity of 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone (HCT) by deferoxamine mesylate (Desferal®) in vivo. Proc Am Assoc Cancer Res 14:18, 1973.
84. ETCUBANAS E, TAN C, WOLLNER N, ET AL. Preliminary clinical trials of 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP). Proc Am Assoc Cancer Res 12:38, 1971.
85. DECONTI RC, CREASEY WA, AGRAWAL KC, ET AL. Clinical pharmacologic studies with 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP). Proc Am Assoc Cancer Res 12:58, 1971.
86. FRENCH FA, and BLANZ EJ, JR. The carcinostatic activity of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone. I. Isoquinoline-1-carboxaldehyde thiosemicarbazone. Cancer Res 25: 1454–1458, 1965.
87. FRENCH FA, BLANZ EJ, JR, DOAMARAL JR, ET AL. Carcinostatic activity of thiosemicarbazones of formyl heteroaromatic compounds. VI. 1-Formylisoquinoline derivatives bearing additional ring substituents, with notes on mechanism of action. J Med Chem 13:1117–1124, 1970.
88. WHEELER GP, BOWDON BJ, ADAMSON DJ, ET AL. Effects of 1,3-bis(2-chloroethyl)-1-nitrosourea and some chemically related compounds upon the progression of cultured H.Ep. No. 2 cells through the cell cycle. Cancer Res 30:1817–1827, 1970.
89. SCHABEL FM, JR. New experimental drug combinations with potential clinical utility. *In* Symposium on Clinical Pharmacology. In press, 1973.
90. GOTTLIEB JA, BAKER LH, QUAGLIANA JM, ET AL. Chemotherapy of sarcomas with a combination of adriamycin and dimethyl triazeno imidazole carboxamide. Cancer 30: 1632–1638, 1972.
91. SUZUKI H, NAGAI K, YAMAKI H, ET AL. On the mechanism of action of bleomycin: scission of DNA strands in vitro and in vivo. J Antibiot 22:446–448, 1969.
92. HAIDLE CW. Fragmentation of deoxyribonucleic acid by bleomycin. Mol Pharmacol 7:654–652, 1971.
93. HAIDLE CW, WEISS KK, and KUO MT. Release of free bases from deoxyribonucleic acid after reaction with bleomycin. Mol Pharmacol 8:531–537, 1972.
94. HORWITZ SB, CHANG C, and GROLLMAN AP. Studies on camptothecin: I. Effects on nucleic acid and protein synthesis. Mol Pharmacol 7: 632–644, 1971.
95. KANN HE, JR, and KOHN KW. Effects of deoxyribonucleic acid-reactive drugs on ribonucleic acid synthesis in leukemia L1210 cells. Mol Pharmacol 8:551–560, 1972.
96. SARTORELLI AC. Combination chemotherapy with actinomycin D and ribonuclease: an example of complementary inhibition. Nature 203:877–878, 1964.
97. VALERIOTE FA, BRUCE WR, and MEEKER BE. Synergistic action of cyclophosphamide and 1,3-bis(2-chloroethyl)-1-nitrosourea on a transplanted murine lymphoma. J Natl Cancer Inst 40:935–944, 1968.
98. SCHABEL FM, JR. Improvement of therapeutic activity of antileukemic agents in experimental systems by varying dose schedules and by combination therapy. *In* Proceedings of the 5th International Congress of Chemotherapy. Vienna, Verlag der Wiener Medizinischen Akademie, 1968.
99. MAYO JG, LASTER WR, JR, ANDREWS CM, ET AL. Success and failure in the treatment of solid tumors. III. "Cure" of metastatic Lewis lung carcinoma with methyl-CCNU (NSC-95441) and surgery-chemotherapy. Cancer Chemother Rep 56: 183–195, 1972.

100. PREUSSMANN A, VON HODENBERG A, and HENGY H. Mechanism of carcinogenesis with 1-aryl-3,3-dialkyltriazenes. Enzymatic dealkylation by rat liver microsomal fraction in vitro. Biochem Pharmacol 18:1–13, 1969.
101. SKIBBA JL, BEAL DD, RAMIREZ G, ET AL. *N*-Demethylation of the antineoplastic agent 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide by rats and man. Cancer Res 30:147–150, 1970.
102. SHEALY YF. Syntheses and biological activity of 5-aminoimidazoles and 5-triazenoimidazoles. J Pharm Sci 59:1533–1558, 1970.
103. WHEELER GP. Mechanism of action of alkylating agents: nitrosoureas. *In* Antineoplastic and Immunosuppressive Agents (Handbook of Experimental Pharmacology) (Sartorelli AC, and Johns DG, eds). Heidelberg, Springer-Verlag, vol 1. In press, 1973.
104. BROCKMAN RW, SHADDIX SC, WILLIAMS M, ET AL. Collateral sensitivity of arabinosylcytosine-resistant leukemia L1210 to deazauridine. Proc Am Assoc Cancer Res 14:16, 1973.
105. HUTCHISON DJ. Cross-resistance and collateral sensitivity studies in cancer chemotherapy. *In* Advances in Cancer Research (Weinhouse S, and Haddow A, eds). New York, Academic Press, 1963, vol 7, pp 235–350.
106. HUTCHISON DJ. Studies on cross-resistance and collateral sensitivity (1962–1964). Cancer Res 25: 1581–1595, 1965.

Biochemical Rationales for the Selection of Combinations of Chemotherapeutic Agents

Vincent H. Bono, Jr.[1]

The problems that an investigator faces when he attempts to select useful combinations of agents on the basis of therapeutic activity in a tumor-bearing animal are many and have been discussed at length. Without any additional information, the number of possible combinations to select from is very large. For this reason, it would be desirable if certain principles could be developed to guide in the selection of combinations for animal experimental trials.

These principles may be concerned with lethal effects on cells as they progress through the proliferative cell cycle and with effects on biochemical processes. Since these kinds of observations are best made in uniform populations of cells, cell culture is an ideal system to use in a study of this nature. However, this system gives very little information about therapeutic activity, and for this reason a direct test of the therapeutic activity of a combination in a tumor-bearing animal is needed.

My objective in this paper is to describe certain biochemical rationales that might be useful for the selection of combinations of agents. Since there are some similarities between rationales based on effects of an agent on a cell as it progresses through the proliferative cell cycle and rationales based on biochemical processes, I will mention two of them briefly (fig 1). One such rationale is to combine two drugs that have their maximal lethal effects on cells which are in different phases of the proliferative cell cycle. A second is to combine an agent which slows the transit of cells through a particular phase of the cycle (though not necessarily having a lethal effect) with a second agent that is maximally lethal in that same phase. This rationale needs to be considered carefully however, since the lethal activity of the second agent may depend on a normal rate of transit through the phase, rather than a reduced one. I will return to this point later with regard to the slowing of a cell's progress through the S phase.

Now let us consider some rationales for selecting agents with known primary or direct interaction effects, in particular, agents that affect nucleic acid metabolism (fig 2). The major actions described for such agents fall into three classes: (*a*) interference with the synthesis of nucleotides and depletion of the substrates in the cell needed for RNA and DNA synthesis; (*b*) substituting for normal nucleotides and being incorporated into DNA or RNA; and (*c*) interacting directly with the structure of nucleic acids and interfering with their function or processing. In addition, the first and third classes may be due to either reversible or irreversible interaction effects. Using these classifications, one rationale could be to combine agents having different kinds of direct interaction effects (fig 3). A second rationale could be to choose an agent which on the basis of its own direct activities might be expected to increase the activation, direct interaction activity, or incorporation of a second agent.

Several years ago, Dr. Robert Handschumacher reported (1) that it is not advantageous to use combinations of agents with similar kinds of direct interaction effects. The agents he studied were the kind that interfered with nucleotide production.

Another kind of direct interaction effect identified for some agents involves the microtubule in the mitotic spindle. The basic molecular biology of this process is not well understood, but some steps are outlined in figure 4. Microtubular protein subunit molecules are synthesized and then assembled by polymerization into microtubules which then function in mitotic spindles and in cilia. The microtu-

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1. Combine two drugs having their maximal lethal effects on cells in different phases of the proliferative cell cycle.

2. Combine an agent which slows transit of cells through a particular phase of the cycle (not necessarily a lethal effect) with a second agent that is maximally lethal in that same phase.

FIGURE 1.—Rationales for selecting drug combinations based on the effect of an agent on a cell as its passes through the proliferative cell cycle.

1. Interference with the synthesis of precursor nucleotides by reversible or irreversible interaction effects.

2. Substituting for normal nucleotides and being incorporated into DNA or RNA.

3. Directly interacting (reversibly or irreversibly) with the structure of nucleic acids.

FIGURE 2.—Major direct interaction effects of agents affecting nucleic acid metabolism.

1. Combine pairs of agents with different direct interaction effects.

2. Combine an agent that increases the activation, direct interaction activity or incorporation of a second agent, with the second agent.

FIGURE 3.—Rationales for selecting drug combinations based on their direct interaction effects on nucleic acid metabolism.

bules act in an unknown way to produce karyokinesis or ciliary motion. Several chemotherapeutic agents (vincristine, vinblastine, and demecolcine)[2] cause depolymerization of the microtubules into their subunits (2,3). Combining agents that affect nucleic acid metabolism with those that interfere with mitotic spindle function should be considered. Parenthetically, there is a need to examine active agents with an unknown primary activity for their ability to interfere with the assembly of microtubular protein subunits into microtubules. Agents that appear to prevent cells from leaving G_2 may be having such an effect.

Figure 5 shows a summary scheme for selecting combinations of agents based on biochemical characteristics. The following steps are involved: (1) identify the direct interaction activities of an agent, and (2) combine agents with different kinds of direct interaction activities or (3) combine agents that might be expected to increase the direct interaction activities of other agents. Having selected a combination then (4) determine the cytocidal characteristics in cell culture and (5) assay for increased therapeutic activity in a tumor-bearing animal system. In each case the rationale for the combination may be tested for the ability to predict increased therapeutic activity.

There is, however, another approach. This approach requires a more complete knowledge of the links between the direct interaction effects of an agent (and they may be several) and the subsequent death of the cell. In other words, what is needed is a body of information which describes the secondary biochemical responses of the cell to the primary effects of the drug action and relates them carefully to the survival or death of the cell. We know, considering the extreme case, that if a drug kills a cell not only won't it proliferate but ultimately it will lose its ability to maintain its structural integrity and will lyse. Thus, one can easily imagine all kinds of biochemical sequelae to drug action. However, there are conditions in which a drug exposure is not lethal and in which some direct interaction effects and some secondary effects can be measured.

Figure 6 attempts to depict the concept I am developing. The objective is to describe a pattern of effects at sublethal conditions and

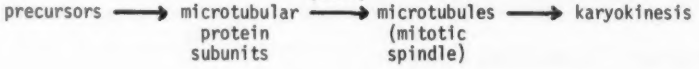

FIGURE 4.—Some steps in the process of mitotic spindle function.

[2] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

Demecolcine: NSC-3096; CAS reg. No. 477-30-5; colchicine, *N*-deacetyl-*N*-methyl-.

1. Identify the direct interaction activities of an agent.
2. Combine agents of different kinds of direct interaction activities.
3. Combine agents that might be expected to increase the direct interaction activities of other agents.
4. Determine the cytocidal characteristics of the combination in cell culture.
5. Determine the therapeutic activity of the combination in tumor bearing animals.

FIGURE 5.—Summary scheme for selecting combinations of agents based on biochemical characteristics.

the pattern of onset of effects under lethal conditions. These sorts of data may allow the organization of drugs into classes based on the pattern of secondary effects rather than on the detailed nature of the primary interaction effects. They may also indicate which of the secondary effects are associated with a lethal effect and which of the effects are reversible. I emphasize the need for a close correlation of the secondary effects to the viability of the affected cells.

A similar presentation of this approach is described by Dr. Emmanuel Farber in *Biochemical Pharmacology* (4) in which he emphasizes the need to describe the response of cells to selective inhibition of DNA, RNA, and protein synthesis (table 1). He points out that protein synthesis inhibitors inhibit cells in early G_2 and that some cause polysome disaggregation and some do not. Agents that inhibit RNA synthesis by binding to DNA produce nucleolar segregation, while agents that inhibit RNA synthesis by other means cause nucleolar fragmentation. Agents that selectively inhibit DNA synthesis do not necessarily have lethal activity; continued protein synthesis seems to be required to produce a lethal effect.

TABLE 1.—Response of cells to inhibition of synthesis of DNA, RNA, and protein*

Selective inhibition	Cell response
Protein synthesis	Interruption of cell cycle in early G_2 with or without disaggregation of polysomes.
RNA synthesis	
a. DNA binding agents	Rapid dissociation of nucleolar components. Slower disaggregation of free polysomes.
b. Others	Fragmentation of nucleolus.
DNA synthesis	Alone not sufficient to produce cell death; requires protein synthesis in addition.

*From data of E. Farber (4).

Figure 7 shows the kinds of secondary effects that can be examined in relation to cell death. The list is both long and incomplete but it serves to indicate the kinds of patterns of drug action that might be observed.

As an example of the application of this approach, I would like to present some data that bears on the problem of defining the conditions that lead to cell death and those that do not lead to cell death when L1210 cells in culture are exposed to hydroxyurea (table 2).[3] It has been shown by several investigators that active DNA synthesis is required for the early lethal action of hydroxyurea. In

[3] Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1.

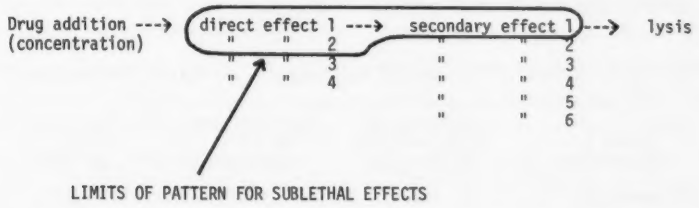

FIGURE 6.-Time course of drug action in terms of effects on biochemical processes.

Ultrastructural Changes	Changes in subcellular organelles.
Energy Metabolism	O_2 utilization and function of Crabtree effect; NAD/NADH, ATP levels.
Gross Metabolism synthesis	Synthesis of DNA, RNA, Protein, Lypoprotein membranes.
Structural Changes in DNA	Occurrence of nicks (single and double strand); extent of methylation; degradation of preformed DNA.
RNA Metabolism	Ribosomal precursor: synthesis, methylation and processing. Heterogeneous nuclear: synthesis, size distribution. Mature ribosomal: assembly of subunits.
Protein Synthesis	Cytoplasmic: synthesis and size distribution. Nuclear: synthesis of histone and acidic protein.

FIGURE 7.—Secondary effects that can be examined in relation to time of onset and in relation to cell death.

this experiment, early stationary stage cells, in which the DNA synthesis rate is only 1% of the mid-log phase rate, are resistant to the action of hydroxyurea for 4 hours. Since this population contains at least 19% cells with an S-phase DNA content, there is an indication that having an S-phase DNA content per se is not sufficient to qualify for a lethal effect.

Since it is clear that the inhibition of DNA synthesis is lethal, how important is the *rate* of inhibition of DNA synthesis (table 3)? To answer this question we compare the effect of adding hydroxyurea all at once (rapidly raising the concentration to 0.5 mM and rapidly reducing DNA synthesis to 2%) to that of gradually increasing concentrations over a 4-hour period to a final concentration of 1.0 mM gradually reducing the DNA synthesis rate to 4% of control. When DNA synthesis is reduced rapidly there is cell death, while when it is reduced slowly over a 4-hour period the cells survive. It might be concluded that it is not the interruption of DNA synthesis that is lethal but the rapidity with which it occurs.

Table 4 shows the effects on the synthesis of nuclear protein when DNA synthesis is promptly inhibited leading to cell death. Histone protein synthesis falls but histone continues to be produced in the absence of new DNA synthesis. There is no effect on nuclear acidic protein or on whole cell protein synthesis. The role of protein synthesis in the production of cell death is suggested in the experiment shown in table 5. Cycloheximide[4] alone (which totally inhibits protein synthesis) is not lethal for 2 hours but prevents the lethal effects of hydroxyurea for 1 hour. This effect is transient however, since with longer exposures cycloheximide itself is lethal.

[4] Cycloheximide: NSC-185; CAS reg. No. 7006-21-5; glutarimide, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-.

TABLE 2.—Effect of 0.5 mM of hydroxyurea on L1210 cell survival

Phase of cell growth	Percent of S-phase cells* (minimum)	Relative rate of DNA synthesis	Surviving fraction (4 hrs)
Exponential	44	[100]	0.55
Early stationary	19	1.2	1.15

*Percent of S-phase cells based on DNA content of cells.

TABLE 3.—Effect of rate of increase of hydroxyurea concentration on L1210 cell survival

	Time (hrs)	Hydroxyurea concentration (mM)	DNA synthesis (% of control)	Surviving fraction
Single addition	1	1.0	2.2	0.59
	2	1.0	2.2	0.50
	3	1.0	1.8	0.36
	4	1.0	1.8	0.31
Multiple addition	1	0.045	75.5	1.26
	2	0.11	40.0	1.12
	3	0.24	19.0	1.00
	4	0.50	9.8	0.93
	5	1.00	4.1	0.93
	6	1.00	6.1	0.90
	7	1.00	7.5	0.71
	8	1.00	9.8	0.89

TABLE 4.—Secondary effects of hydroxyurea on whole cell and nuclear protein production

Time interval (hrs)	Rate of synthesis*			
	Histone	Nonhistone	Whole cell	DNA
0–1.5	51	88	101	6
1.5–3.0	49	89	95	7

*Relative to the rate of untreated controls.

TABLE 5.—Effect of cycloheximide on cell kill activity of hydroxyurea

Condition	Surviving fraction		
	0 hr	1 hr	2 hrs
0.5 mM hydroxyurea at 1 hr	1.00	1.00	0.77
1 μg/ml cycloheximide at 0 hr	1.00	0.92	0.88
Cycloheximide at 0 hr and hydroxyurea at 1 hr	1.00	0.95	0.95

These studies on the relationship of protein synthesis to the lethal activity of hydroxyurea are incomplete. Additional studies of other biochemical changes in response to hydroxyurea are needed to further define the biochemical consequences of this drug action.

How then could this information be used in selecting drug combinations? Perhaps agents that prolong S phase and reduce the rate of synthesis of DNA should not be used with hydroxyurea. Agents that slow the rate of synthesis of protein might also be considered a poor choice. One might speculate that an agent that stimulates protein synthesis might increase the lethal effects of hydroxyurea. Data of this kind are not yet complete but with additional studies a rational basis for combining agents may become apparent.

REFERENCES

1. HANDSCHUMACHER RE. Formal discussion: some enzymatic considerations in combination chemotherapy. Cancer Res 25:1541–1543, 1965.
2. MALAWISTA SE, SATO H, and BENSCH KG. Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. Science 160:770–772, 1968.
3. BANERJEE S, and MARGULIS L. Inhibition of cilia regeneration by antineoplastic agents. Cancer Chemother Rep 55:531–537, 1971.
4. FARBER E. Response of cells to inhibition of synthesis of DNA, RNA and protein. Biochem Pharmacol 20:1023–1026, 1971.

Combination Therapy: Some Concepts and Results [1]

Howard E. Skipper [2]

Some time ago I was talking with Dr. Leon H. Schmidt about the successes and failures in chemotherapy for tuberculosis and malaria—two of the fields that he has contributed to over many years. Several comments he made about the remarkable progress in the cure of tuberculosis have stuck in my mind:

1. Today, one would never start out treating advanced TB with a single drug because of the drug-resistance problem.
2. Combinations of drugs do indeed provide higher cure rates.
3. The combination of an anti-TB drug, which is relatively poor by itself, with a better drug will improve on the therapeutic results obtainable with the better drug used alone.

I wish we had anticancer drugs with therapeutic indices such as isonicotinic acid hydrazide for TB or penicillin for other bacterial diseases. We don't. Nevertheless, the value of combination chemotherapy in the treatment of disseminated animal and human cancers has now been established beyond reasonable doubt (1).[3] Some early animal cancers and perhaps two types of disseminated human cancer have been "cured" by optimal use of single drugs. However, combination chemotherapy usually has been required for the best responses in *advanced* animal or human cancers.

Also, it has been demonstrated that surgical removal of major solid tumor masses increases the chances of chemotherapeutic cure of certain disseminated cancers (ie, where definitive therapeutic trial results are available) (2–10).

We suspect that the advantages of combination chemotherapy and surgery-chemotherapy for cancer are associated with several phenomena:

1. *The drug-resistance problem.*—It is not unusual for one in 10^6–10^7 bacterial or cancer cells to be resistant to single drugs. In fact, the mechanisms by which cancer cell populations become resistant to some drugs is now well understood (11). Combinations of certain drugs lessen the emergence of true [4] drug resistance. Also, surgical removal of solid tumor masses may, in some instances, leave relatively small numbers of widely disseminated tumor stem cells, eg, less than 10^6–10^7.

2. *Selectivity.*—Combinations of drugs have been found which are less than additive in host toxicity but additive (or greater than additive) with respect to tumor cell kill (1,12).[3] Since host toxicity is about "half of our problem," such discoveries may represent important "therapeutic potentiation."

[1] Much of the data presented herein were gained under contract NIH-NCI-C-71-2098 from the Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare (by my associates Drs. Schabel, Schmidt, Griswold, Laster, Mayo, Trader, and their associates). The concepts expressed have been building for years as a result of the exchange of data and views between our Organic Chemistry, Biochemistry, Pharmacology, and Chemotherapy Departments, and our preclinical and clinical colleagues at the NCI and institutions in this country and abroad. Our Biochemistry Department has been exceptionally helpful in suggesting useful combination chemotherapy.

[2] Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.

[3] Schabel FM, Jr. New experimental drug combinations with potential clinical unity. Presented at the Symposium on Clinical Pharmacology (sponsored by the Cancer Clinical Investigation Review Committee, Clinical Investigations Branch, Division of Cancer Grants, NCI), Jacksonville, Fla, Mar 1973.

[4] True drug resistance is resistance in a biochemical sense; eg, after continuing treatment of tumor-bearing animals with purine or pyrimidine antagonists, tumor cell populations overgrow that have lost the specific enzymes required for activation of the antagonists (11). Such resistance should not be confused with the simple inability of drugs to kill sensitive cancer cells as fast as they proliferate. In therapeutic trials one usually does not know whether failure, in the face of continuing therapy, is the result of drug resistance in the biochemical sense, or inappropriate dosage and schedule, or a bit of both.

3. *Quantitative biochemical-cytokinetic characteristics* [5] *(in large solid tumor masses versus small micro- or macro-colonies of tumor cells missed at surgery).*—From the standpoint of chemotherapeutic eradication, the degree of biochemical-cytokinetic heterogeneity [5] appears greater (and thus less favorable) in solid tumor masses than in very small metastatic colonies (5,13). Thus, surgical removal or radiotherapeutic ablation of local tumor masses may improve chances for chemotherapeutic eradication of residual disease, if it also is not too advanced (5).

I will present several illustrations of the types of data which led me to the views expressed above.

I. DRUG RESISTANCE

Table 1 presents data showing that, in a system we have worked with a great deal, we cannot cure animals with arabinosylcytosine [6] (ara-C) alone when there are 10^7 or more leukemia cells present at initiation of treatment. Good increases in survival time are achieved, but animals eventually die even during continuing treatment and with typical widespread disease. The data presented in table 2 show that a leukemia cell population that survives four or more courses of ara-C is indeed resistant.

Table 3 summarizes results obtained with animals bearing about 10^8 or 10^7 leukemia cells at initiation of treatment with combinations of ara-C plus 6-thioguanine.[7] This is a rather striking quantitation demonstration of thera-

[5] These characteristics refer to the relative distributions of tumor stem cell residence times in G_1, or if you prefer biochemical terms, the relative fractions of viable tumor cells initiating DNA replication per unit time. Many, perhaps most, of our anticancer drugs kill cells as a result of their effects on DNA replication or function (13,14).

[6] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[7] Burchenal first observed that ara-C plus 6-mercaptopurine was potentiating in mouse leukemia. Clarkson then showed that ara-C plus 6-thioguanine was an effective combination in human leukemias (15). The data in table 3 are unpublished results of my associate, Dr. Frank M. Schabel, Jr.

Table 1.– Depending on the tumor cell population size at initiation of treatment, true drug resistance may or may not underlie failure of single drugs (L1210 leukemia model).

Site and Size of Inoculum	Ara-C; tolerated doses q3hrs (x 8) on the days indicated *	No. of Courses	% ILS (excluding "cures")	% "Cures"	Estimated average number of leukemia cells surviving at end of R_x †	Comment
IP; 10^4	2 only	1	63	50	1	About 10^5 leukemic cells at initiation of R_x. Drug-resistance was not limiting with respect to "cure"
IV; 10^4	2, 6, 10 and 14	4	~	84	<1	
IP; 10^5	2 only	1	89	0	10	About 10^6 leukemic cells at initiation of R_x. Failure of single course not due to resistance—see 2, 3, and 4 courses.
	2 and 6	2	156	32	1	
	2, 6 and 10	3	~	90	<1	
	2, 6, 10 and 14	4	~	90	<1	
IV; 10^6	2, 6, 10 and 14	4	300	0	10^3	About 10^7 leukemic cells at initiation of R_x. Failure due to selection and overgrowth of ara-C resistant leukemic cells. ¶
	2, 6, 10, 14, 18 and 22	6	400	0	10^5	
	Every 4th day, 2-30	8	491	0	10^7	
	Every 4th day, 2-38	10	533	0	10^8 ‡	
	Every 4th day, 2-46	12	508	0	10^9 §	

* Normal animals survived the above therapeutic regimens with no weight loss.

† Based on median survival time after end of R_x and "cures".

‡ Some animals died during R_x.

§ All animals died during R_x.

¶ When leukemic cells were harvested from mice which received 4 or more courses of treatment and passed to other animals, the passage lines failed to respond to ara-C (see Table 2).

Table 2.– Evidence that the failure of ara-C may result from true drug resistance*

L1210 Leukemia Cells	Site and Size of Inoculum	Ara-C, Dosage and Schedule	% ILS	% "Cures"
Original line	IV; 10^7	15 mg/kg, q3hr (x 8), q4d (x 4)	212	0
Passage after ara-C R_x †	IV; 10^7	15 mg/kg, q3hr (x 8), q4d (x 4)	14	0
Original line	IV; 10^6	15 mg/kg, q3hr (x 8), q4d (x 4)	409	0
Passage after ara-C R_x	IV; 10^6	15 mg/kg, q3hr (x 8), q4d (x 4)	12	0
Original line	IV; 10^5	15 mg/kg, q3hr (x 8), q4d (x 4)	314	20
Passage after ara-C R_x	IV; 10^5	15 mg/kg, q3hr (x 8), q4d (x 4)	5	0

* These are data of Dr. F. M. Schabel, Jr.

† This ara-C-resistant line was obtained from pooled spleens of 4 animals after they had received 4 courses of ara-C. These animals were bearing about 10^7 i. v. inoculated leukemia cells at initiation of treatment.

This cell line was as sensitive as the parent line to another S-phase specific agent, pyridine-2-carboxaldehyde thiosemicarbazone, which is an inhibitor of nucleotide reductase rather than DNA polymerase.

peutic potentiation that has been readily reproducible. We believe that the ara-C plus 6-thioguanine combination has two advantages in the treatment of leukemias: (*a*) the combination reduces failures due to drug resistance, and (*b*) the combination is much less than additive in toxicity to the host.

II. LESS-THAN-ADDITIVE, ADDITIVE, AND GREATER-THAN-ADDITIVE TOXICITY OF COMBINATIONS

Frei has pointed out that one of the rationales employed in seeking therapeutically potentiating combinations of drugs is to select agents with differing types of toxicity to the

Table 3.—Therapeutic potentiation of the ara-C + 6-thioguanine combination (L1210 leukemia model)

Site and Size of Inoculum*	Treatment	mg/kg/dose	Schedule; 1st course on day 2	% ILS (excluding "cures")	% "Cures"
IV; 10^7	Ara-C	15	q3hrs (x 8), q4d (x 4)	350	0
		10	q3hrs (x 8), q4d (x 4)	388	0
		6.7	q3hrs (x 8), q4d (x 4)	288	0
IV; 10^7	6-Thioguanine	0.67 (tox)†	q3hrs (x 8), q4d (x 4)	100	0
		0.45	q3hrs (x 8), q4d (x 4)	50	0
		0.30	q3hrs (x 8), q4d (x 4)	25	0
IV; 10^7	Ara-C + 6-TG	15 + 0.67 (tox)	q3hrs (x 8), q4d (x 4)	588	10
		15 + 0.45	q3hrs (x 8), q4d (x 4)	600	0
		15 + 0.30	q3hrs (x 8), q4d (x 4)	500	10
		10 + 0.67	q3hrs (x 8), q4d (x 4)	525	0
		10 + 0.45	q3hrs (x 8), q4d (x 4)	475	0
		10 + 0.30	q3hrs (x 8), q4d (x 4)	425	0
IV; 10^6	Ara-C	15	q3hrs (x 8), q4d (x 4)	460	0
		10	q3hrs (x 8), q4d (x 4)	360	0
		6.7	q3hrs (x 8), q4d (x 4)	320	0
IV; 10^6	6-Thioguanine	0.67 (tox)	q3hrs (x 8), q4d (x 4)	90	0
		0.45	q3hrs (x 8), q4d (x 4)	60	0
		0.30	q3hrs (x 8), q4d (x 4)	40	0
IV; 10^6	Ara-C + 6-TG	15 + 0.67 (tox)	q3hrs (x 8), q4d (x 4)	780	60
		15 + 0.45	q3hrs (x 8), q4d (x 4)	660	60
		15 + 0.30	q3hrs (x 8), q4d (x 4)	520	10
		10 + 0.67	q3hrs (x 8), q4d (x 4)	660	60
		10 + 0.45	q3hrs (x 8), q4d (x 4)	520	0
		10 + 0.30	q3hrs (x 8), q4d (x 4)	440	0

* The number of leukemia cells at initiation of R_x would be about 1 decade higher.

† Toxic in concurrent toxicity control animals, i. e., mortality of 2/10 or >.

Note: (1) These are results from a large, single, internally controlled trial in which all animals received inocula from the same population of leukemia cells prior to randomization into control and treatment groups.

(2) The ara-C + 6-thioguanine combination has two advantages: (a) in reducing failures due to drug resistance, and (b) the fact that the combination is less than additive in host toxicity.

host, ie, combinations which are less than additive with respect to host toxicity and with additive or greater-than-additive tumor cell kill (1). This approach has been productive.

Not long ago we developed a reasonably quantitative method for examining the toxicity of combinations of drugs in our most used "model" host, the mouse. The question we are asking is: Are two (or more) drugs that have been evaluated for therapeutic potentiation—or are being considered—less than additive, additive, or more than additive with respect to toxicity? We know that the mouse is a reasonable quantitative predictor of toxicity for man (16). A description of the method and results obtained follows.

a. The doses of individual drugs used in a combination toxicity study were tabulated in terms (decimal fractions) of the previously determined LD10s for the same schedule.

b. The decimal fractions of the LD10s of the individual agents were added and the sums plotted against observed mortality on log-probit paper.

c. The LD10 (sum) of the combination was then read from a line of best fit. All animals were observed for 45 days or longer after the end of treatment. Example:

		LD_{10} (mg/kg)	Decimal Fraction of LD_{10}		Interpretation re Toxicity
			Individual Agents	Sum	
	Drug A	100	1.0	—	—
	Drug B	100	1.0	—	—
(a)	Drug A + B	50 + 50	0.5 + 0.5	1.0	Additive
(b)	Drug A + B	100 + 100	1.0 + 1.0	2.0	Less than additive
(c)	Drug A + B	25 + 25	0.25 + 0.25	0.5	More than additive

Note: In the plots and tabulations which will be presented, the ratios of Drug A and Drug B were not always 1:1 with respect to fractions of the LD_{10}'s of individual agents.

Figure 1 shows that when the sums of the decimal fractions of the LD10s of ara-C and 6-thioguanine are plotted against observed mortality, the combination toxicity is *less than additive* as reported by Schmidt et al (12). Each point represents a group of ten BDF_1 mice. In this instance inspection of tabulated data already had led to the conclusion that the

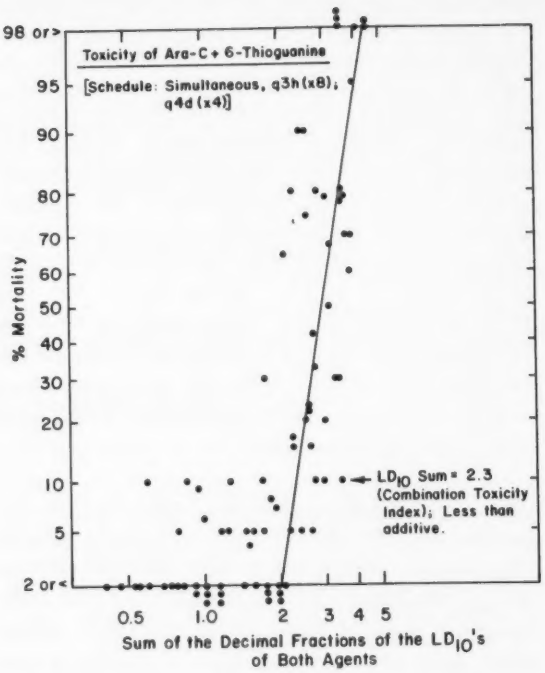

FIGURE 1.—The toxicity of ara-C + 6-thioguanine to BDF_1 mice. An illustration of a combination which is much less than additive in toxicity. This combination is therapeutically potentiating.

toxicity of this combination was far less than additive. However, with other combinations which are closer to additive in toxicity, a quantitative conclusion is much more difficult on quick perusal of tabulated dose-mortality data.

Figure 2 shows that the toxicity of CCNU plus BIC (NSC-82196) when given as a single dose is approximately additive. This combination, suggested by Dr. Glynn P. Wheeler on biochemical grounds, has been shown to be therapeutically potentiating against the L1210 leukemia model.[8]

Figure 3 shows the surprising greater-than-additive toxicity of CCNU plus DIC (NSC-45388). This combination has shown no evidence of therapeutic potentiation.[8]

The available combination toxicity data on some 40 pairs of drugs have been plotted in the manner indicated above. The results are listed in table 4. As indicated in the footnote

[8] *See* footnote 3.

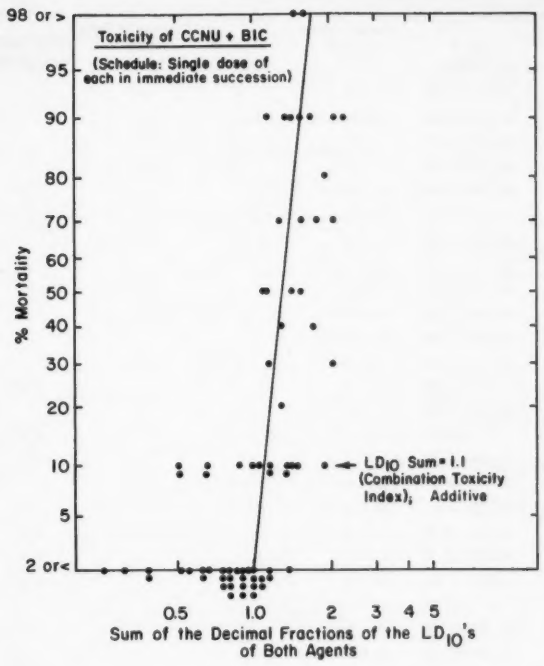

FIGURE 2.—The toxicity of CCNU + BIC to BDF_1 mice. An illustration of a combination which is additive in toxicity. This combination is therapeutically potentiating.

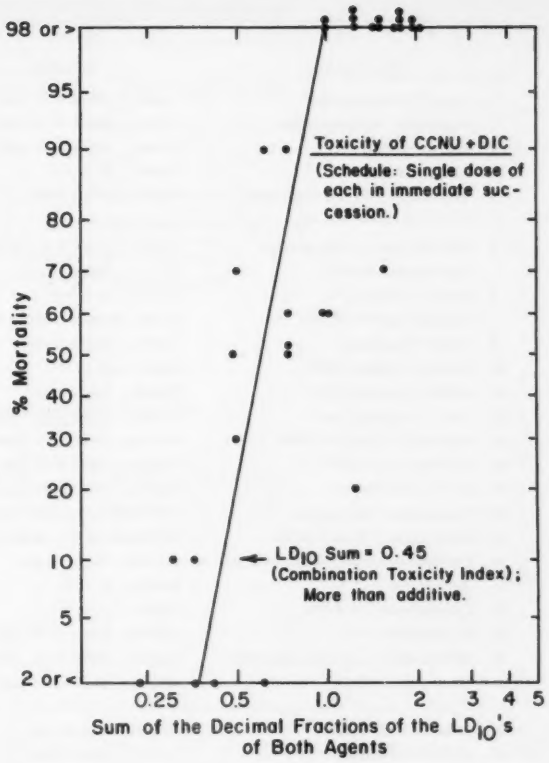

FIGURE 3.—The toxicity of CCNU + DIC to BDF_1 mice. An illustration of a combination which is more than additive in toxicity. This combination is not therapeutically potentiating.

to table 4, some of the values require confirmation and we cannot be certain that similar values would result if quite different schedules were employed.

Within the sample of two-drug combinations examined (table 4), I must admit to some surprise that about half of them were less than additive in toxicity (*see* table 5). The base-line data (individual drug LD10s) were from pooled and, in most instances, very extensive past experience.

I am of the opinion that analyses of combination toxicity data in the manner just described will be helpful to us in future efforts to select combinations, design the most efficient combination chemotherapy trials, and interpret the results obtained in such trials. In addition, we will require quantitatively comparable information on the rate of recovery from toxicity of our model host and man (single agents and combinations) before we can translate with confidence.

SURGERY-CHEMOTHERAPY

The following are examples of the advantages of surgery-chemotherapy. Karrer, Humphreys, and Goldin (2–4) have shown that surgery followed by cyclophosphamide will increase the cure rate of metastatic Lewis lung carcinoma in the mouse. Farber (8) has reported that surgery and radiotherapy followed by actinomycin D markedly improves the long-term tumor-free survivors in children with Wilms' tumor over that obtainable with single therapeutic modalities.

The data presented in table 6 and figure 4 show the "cure" rates of increasingly advanced metastatic Lewis lung carcinoma when surgery is followed by single drugs or a combination which has been shown to be therapeutically

Table 4.– On the toxicity of pairs of drugs given in combination (BDF_1 mice)

	Combination	Schedule	Log P Values	Combination Toxicity Index	Classes of Drugs: S-phase Specific	S-phase Self-limiting	Cycle-phase nonspecific
1.	Ara-C + 6-Thioguanine	Simult., q3hrs (x 8); q4d (x 4)	-2.11; -0.06	2.3	✓	✓	
2.	Gunazole + 6-Thioguanine	Simult., q3hrs (x 8); q4d (x 3)	-1.61; -0.06	2.0 or >	✓	✓	
3.	Ara-C + 6-MP	Simult., q3hrs (x 8); q4d (x 3)	-2.11; +0.01	2.0	✓	✓	
	Ara-C + 6-MP	Simult., qd (x 15)	-2.11; +0.01	1.7	✓	✓	
4.	Vincristine + Cyclophosphamide	Simult., Single doses	+0.63; +2.82	ca 2 (?)			
5.	Hydroxyurea + 6-Thioguanine	Simult., q3hrs (x 8); q4d (x 4)	-1.80; -0.06	1.9	✓	✓	
6.	Vincristine + BCNU	Simult., Single doses	+2.82; +1.53	ca 1.9 (?)			✓
7.	6-MP + Daunomycin	Simult., qd (x 15)	+0.01; +1.83	1.8		✓	✓
8.	6-Methyl MPR + BCNU	Offset, qd (x 15); single dose	+0.05; +1.53	ca 1.8 (?)		✓	✓
9.	5-FU + Prednisone	Simult., Single doses	-0.89; +1.46	ca 1.7 (?)			
10.	Ara-C + 6-Methyl MPR	Simult., qd (x 15)	-2.11; +0.05	1.6	✓	✓	
11.	BCNU + Methyl-CCNU	Simult., Single doses	+1.53; +3.30	1.6			✓ ✓
12.	Ara-C + Hydroxyurea	Simult., q3hrs (x 8); q4d (x 4)	-2.11; -1.80	1.6	✓ ✓		
13.	Bleomycin + Methyl-CCNU	Overlap, qd (x 10) + days 7 & 11	? ; +3.30	1.6			✓ ✓
14.	Hydroxyurea + BCNU	Simult., q3hrs (x 8); q4d (x 4)	-1.80; +1.53	1.6	✓		✓
15.	5-FU + Vincristine	Simult., Single doses	-0.89; +2.82	ca 1.6 (?)			
16.	Vincristine + Bleomycin	VCR 6 hr before Bleo, qd (x 10)	+2.82; ?	ca 1.6 (?)			
17.	Adriamycin + Methyl-CCNU	Offset, qd (x 8) + single dose	+1.27; +3.30	1.5			✓ ✓
18.	Cyclophosphamide + Methyl-CCNU	Simult., Single doses	+0.63; +3.30	1.5			✓ ✓
19.	Adriamycin + DIC	Simult., qd (x 8)	+1.27; -0.24	ca 1.5			✓ ✓
20.	Cis-platinum II + 5-FU	Simult., qd (x 5)	? ; -0.89	1.5			
21.	Hydroxyurea + DIC	Overlap, q3hrs (x 8) + qd (x 5)	-1.80; -0.24	ca 1.5	✓		✓
22.	Hydroxyurea + Cyclophosphamide	Simult., q3hrs (x 8), q4d (x 4)	-1.80; +0.63	ca 1.5 (?)	✓		✓
23.	Bleomycin + CCNU	Overlap, qd (x 10) + days 3 & 10	? ; +2.83	ca 1.5 (?)			✓ ✓
24.	Methyl-CCNU + BIC	Simult., Single doses	+3.3; +1.54	1.4			✓ ✓
25.	BCNU + Prednisone	Simult., Single doses	+1.53; +1.46	ca 1.4 (?)			
26.	BCNU + Cyclophosphamide	Simult., Single doses	+1.53; +0.63	1.2-1.4			✓ ✓
27.	Ara-C + BCNU	Simult., q3hrs (x 8), q4d (x 4)	-2.11; +1.53	1.3			✓ ✓
28.	6-MP + 6-Methyl MPR	Simult., qd (x 15)	+0.01; +0.05	1.3		✓ ✓	
29.	Ara-C + CCNU	Simult., q3hrs (x 8), q4d (x 4)	-2.11; +2.83	1.3	✓		✓
30.	Ara-C + Guanazole	Simult., q3hrs (x 8), q4d (x 4)	-2.11; -1.61	1.2	✓ ✓		
31.	BCNU + CCNU	Simult., Single doses	+1.53; +2.83	1.2			✓ ✓
32.	5-FU + BCNU	Simult., Single doses	-0.89; +1.53	ca 1.2 (?)		✓	✓
33.	6-Thioguanine + DIC	Simult., qd (x 15)	-0.06; -0.24	ca 1.2 (?)		✓	✓
34.	Cyclophosphamide + BIC	Simult., Single doses	-0.63; +1.54	ca 1.2 (?)			✓ ✓
35.	CCNU + BIC	Simult., Single doses	+2.83; +1.54	1.1			✓ ✓
36.	CCNU + L-PAM	Simult., Single doses	+2.83; -0.66	1.1			✓ ✓
37.	Cyclophosphamide + CCNU	Simult., Single doses	+0.63; +2.83	ca 1.1			✓ ✓
38.	6-Thioguanine + 6-Methyl MPR	Simult., qd (x 15)	-0.06; +0.05	1.0		✓ ✓	
39.	Cyclophosphamide + DIC	Simult., Single doses	-0.63; -0.24	ca 1.0 (?)			✓ ✓
40.	BCNU + DIC	Simult., Single doses	+1.53; -0.24	0.75			✓ ✓
41.	CCNU + DIC	Simult., Single doses	+2.83; -0.24	0.45			✓ ✓

Note: ? implies scant data. We cannot be certain that the same relationships will hold for quite different schedules.

potentiating. These are data of Mayo et al (5). They also have demonstrated that widely metastatic micro- or macro-colonies of Lewis lung carcinoma cells are relatively easy to cure by chemotherapy in the absence of a large solid primary (table 7).

Figure 5 shows the advantage of surgery-chemotherapy in the "cure" of B16 melanoma in the mouse. This solid animal tumor is indeed refractory to chemotherapy alone. These are unpublished data of Griswold.

Finally, for theoretical reasons—and even

Table 5.—The frequency of less than additive, additive, and greater than additive toxicity of 2-drug combinations (in the sample studied)

Toxicity	Combination toxicity index	% of 41 different pairs examined	Prevalent classes of drugs
Much less than additive	2 or >	10	S-phase specific + purine antag.
Less than additive	1.5-1.0	46	Varied
Additive or slightly <	1.0-1.5	39	2-cycle-phase nonspecific
Greater than additive	<1.0	5	A nitrosourea + DIC (not BIC)

Note: The observation that 56% of the 41 pairs examined were less than additive in toxicity was somewhat surprising to me—perhaps it was a skewed or unrepresentative sample.

Table 6.—Surgery, chemotherapy, and surgery → chemotherapy (Solid Lewis lung carcinoma; metastatic)

Solid Primary Tumor Wt. (gms)	% Cures						
	Surgery Alone	Chemotherapy Alone			Surgery → Chemotherapy		
		Me-CCNU	Cy	Me-CCNU + Cy	Me-CCNU	Cy	Me-CCNU + Cy
0.4	0	34	0	68	40	–	–
1.0	0	0	0	24	30	35	70
2.0	0	0	0	0	18	15	65
3.0	0	0	0	0	0	0	–

Table 7.—"Cure" of disseminated Lewis lung carcinoma in the absence of a solid primary (10^6 tumor cells inoculated iv)

Agent	Single dose (mg/kg)	% Tumor-free Survivors when a Single Dose was Given on:			
		Day 2	Day 6	Day 10	Day 14
Me-CCNU	36	79	71	40	14
	24	79	46	6	4
	12	26	3	0	7
Cy	312	75	40	45	0
	208	75	84	6	0
	103	26	20	0	0

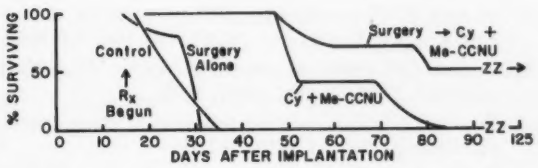

FIGURE 4.—The effects of surgery alone, chemotherapy alone, and surgery followed by chemotherapy on survival time of animals bearing 1-g solid Lewis lung tumors and widely metastatic macro-colonies.

more because of pragmatic therapeutic trial results I have seen—I am encouraged to anticipate the stepwise increase in the control and cure of disseminated cancers by combination chemotherapy and by surgery and/or radiotherapy followed by optimal chemotherapy.

DEFINITIONS

Log P: the lipophilic character of an agent is operationally defined by log P where P is the octanol-water partition coefficient of the drug (17).

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Arabinosylcytosine: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytosine arabinoside; ara-C

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

BIC: NSC-82196; CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-

Bleomycin: NSC-125066

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

***Cis*-diamminedichloroplatinum:** NSC-119875; CAS reg. No. 15663-27-1; *cis*-pt II

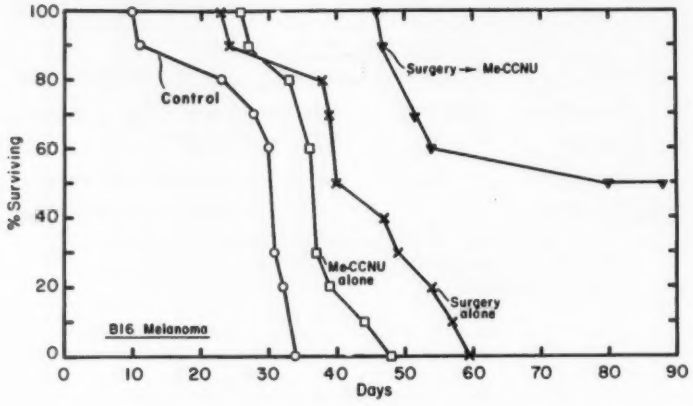

FIGURE 5.—The effects of surgery alone, chemotherapy alone, and surgery followed by chemotherapy on survival time of animals bearing established B16 melanoma (a model system which is unusually refractory to chemotherapy alone).

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Daunomycin: NSC-82151; CAS reg. No. 23541-50-6; daunorubicin (NSC-83142)

DIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Guanazole: NSC-1895; 1,2,4-triazolidine, 3,5-diimino-

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

6-Methylmercaptopurine riboside: NSC-40774; 9*H*-purine, 6-(methylthio)-9-β-D-ribofuranosyl-, dihydrate; 6-methyl MPR

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCES

1. FREI E, III. Combination cancer therapy: Presidential Address. Cancer Res 32:2593–2607, 1972.
2. KARRER K, HUMPHREYS SR, and GOLDIN A. An experimental model for studying factors which influence metastasis of malignant tumors. Int J Cancer 2:213–223, 1967.
3. KARRER K, and HUMPHREYS SR. Continuous and limited courses of cyclophosphamide (NSC-26271) in mice with pulmonary metastasis after surgery. Cancer Chemother Rep 51:439–449, 1967.
4. HUMPHREYS SR, and KARRER K. Relationship of dose schedules to the effectiveness of adjuvant chemotherapy. Cancer Chemother Rep 54:379–392, 1970.
5. MAYO JG, LASTER WR, JR, ANDREWS CM, ET AL. Success and failure in the treatment of solid tumors. III. "Cure" of metastatic Lewis lung carcinoma with methyl-CCNU (NSC-95441) and surgery-chemotherapy. Cancer Chemother Rep 56:183–195, 1972.
6. DEWYS WD. A quantitative model for the study of the growth and treatment of a tumor and its metastases with correlation between proliferative state and sensitivity to cyclophosphamide. Cancer Res 32:367–373, 1972.
7. FUGMANN RA, MARTIN DS, HAYWORTH PE, ET AL. Enhanced cures of spontaneous murine mammary carcinomas with surgery and five-compound combination chemotherapy, and their immunotherapeutic interrelationships. Cancer Res 30:1931–1936, 1970.
8. FARBER S. Chemotherapy in the treatment of leukemia and Wilms' tumor. JAMA 198:154–164, 1966.
9. ZUBROD CG. Chemical control of cancer. Proc Natl Acad Sci USA 69:1041–1047, 1972.
10. ZUBROD CG. The basis for progress in chemotherapy. Cancer 30:1474–1479, 1972.
11. BROCKMAN RW. Mechanisms of resistance. *In* Antineoplastic and Immunosuppressive Agents (Handbook of Experimental Pharmacology) (Sartorelli AC, and Johns DC, eds). In press.
12. SCHMIDT LH, MONTGOMERY JA, LASTER WR, JR, ET AL. Combination therapy with arabinosyl cytosine

and thioguanine. Proc Am Assoc Cancer Res 11:70, 1970.
13. SKIPPER HE. Successes and failures at the preclinical level; where now? *In* Seventh National Cancer Conference Proceedings. Philadelphia, J. B. Lippincott Co., 1973, 968 pp.
14. SKIPPER HE, SCHABEL FM, JR, MELLETT LB, ET AL. Implications of biochemical, cytokinetic, pharmacologic, and toxicologic relationships in the design of optimal therapeutic schedules. Cancer Chemother Rep 54:431–450, 1970.
15. CLARKSON BD, and FRIED J. Changing concepts of treatment in acute leukemia. Med Clin North Am 55:561–600, 1971.
16. FREIREICH EJ, GEHAN EA, RALL DP, ET AL. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster dog, monkey, and man. Cancer Chemother Rep 50:219–244, 1966.
17. HANSCH C, SMITH N, ENGLE R, ET AL. Quantitative structure-activity relationships of antineoplastic drugs: nitrosoureas and triazenoimidazoles. Cancer Chemother Rep 56:443–456, 1972.

Therapeutic Synergism

Nathan Mantel [1]

What constitutes drug synergism has been variously defined at different times and in different places. Some unique property of a particular combination of drugs may underlie a claim for synergism, but the rationale for early drug combinations at times may have been based on a desire to make use of the separate properties of each drug. However, a worthwhile improvement in therapeutic effect could have resulted from some combinations (eg, a combination that affords the rapid action of one ingredient together with the sustained action of another).

Such combinations were involved in an insecticide testing program with which I was once associated. Insecticide combinations being tested involved agents like pyrethrins, which have immediate effects on flies, mosquitoes, and other insects, together with DDT, which has slower but more lethal effects. Both agents were in limited supply at the time (in one case because of wartime shortages and in the other case because of the novelty of the agent), and it is probable that one aim of the program was to stretch limited supplies. This goal of doing more with less is reasonable even if supplies are unlimited since it can lead to cheaper and more competitive products. A second possible advantage of an effective combination would have been that less effort would be required to apply less insecticide. (Present-day insecticides are so potent, however, that they are generally used in mixtures consisting mainly of inert ingredients.) Another advantage of effective combinations that might be appreciated today would be the reduction in the amount of insecticide added to the environment when lesser amounts are used in combination.

This kind of economic synergism between agents does not have complete carryover in the treatment of disease. For the treatment of disease in man, we should be interested in identifying the treatment combinations that give better results (ie, combinations that have therapeutic synergism) rather than the combinations that economize on drug quantities. The two kinds of synergism might both obtain when the use of drugs in combination leads to a reduction in toxicity because of a reduction in the amounts required; however, such a reduction in drug requirements is not a necessary concomitant of therapeutic synergism. Studies on the treatment of disease with drug combinations were frequently carried out using the customary rationale and approach employed in investigations not concerned with disease (such as investigations of insecticide combinations). In early investigations, an economic synergism approach might have been justified even in studying the use of drug combinations for the treatment of disease, because an economic motivation did indeed exist. When penicillin was first introduced, it was limited in supply and expensive. At that time it was reasonable to seek out ways which permitted more sparing use of the drug. Today, however, combinations providing more effective rather than more sparing use of penicillin are the ones to be sought.

The work of Dr. Harry Eagle, former Scientific Director of the National Cancer Institute (NCI), provides an example of application of the economic synergism approach. Dr. Eagle demonstrated synergism between the arsenical mapharsen and the antibiotic bacitracin in the treatment of syphilis in rabbits. (Dr. Eagle, like several other early NCI investigators, graduated from venereal disease research into cancer research.) The combination dose yielding a 50% cure (ED50) of rabbit syphilis contained quantities of each agent that when expressed as fractions of their separate ED50 values and added came to less than unity. Thus, combination treatment would require less material than would treatment with

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each drug alone. (Note, however, that if the total of the fractions is unity [or even more than unity], a condition of partial additivity might exist under which the use of more of an agent that is abundant could result in the sparing use of an agent in critical supply.)

Dr. Eagle was of course aware that his demonstration of synergism was not quite appropriate—he was only using what seemed to be the standard method for demonstrating synergism. The real problem was that neither agent alone yielded 100% success in the treatment of syphilis in man, and the extent to which each agent could be used alone was limited by toxicity or other factors. Dr. Eagle's expressed hope was that synergism (demonstrated in rabbits by reduced drug needs) would be reflected in improved cure rates in man.

We can see that a system directed to determining if the combination of agents would yield higher cure rates for rabbit syphilis than either agent alone might have been a more logical system for Dr. Eagle to use. In any morbidity situation it seems natural (at least when single agents are involved) to see what the problem is and to ask what kind of solution is needed. When all patients (eg, leukemic mice) are dying early, let us try to prolong their survival time. If we are getting some cures, let us try to raise the cure rate—to 100% if possible. If the cure rate is 100% but the treatment period is prolonged, let us try to shorten it. Also, further steps could be taken to develop treatment that can be applied at home by the patient himself so that hospital stays are avoided.

These considerations apply when single drugs are being compared or when modified treatment procedures with a particular drug are being compared. They should also apply when combinations of drugs are being studied since all that has been changed is the possible number of variations in treatment (with two drugs or more, the possible number of variations is enormously increased). However, in studies of drug combinations, investigators are frequently prisoners of published techniques for establishing synergism. Instead of being directed toward the identification of improved therapeutic regimens using drugs in combination, their research follows the lines called for by the techniques. As an additional attraction, these techniques call for much simpler experimental designs and require much less work than would be necessary if the identification of improved therapeutic regimens was the goal.

The demonstration of economic synergism is not, in fact, the only approach generally used, but it is the most common method. There are several alternative methods (which I will not describe in detail here). The common thread the various approaches have is that each requires the postulation of some simple, nonsynergistic model that relates the effects of the drugs in combination to the effects of the separate drugs. If the experimental data do not conform to the model, then this is taken as evidence of drug synergism, drug potentiation, or even drug antagonism, according to the circumstances. This subjects the investigator to the risk that even if the effects of the drugs are in fact simply additive according to one nonsynergistic model, they could seem to be combining in a nonadditive fashion if an alternative incorrect nonsynergistic model were postulated for purposes of analyzing the experimental data. In a survey that Dr. Abraham Goldin (NCI) and I made of various studies of drug combinations in the treatment of experimental cancer, we found that investigators frequently seemed to select nonsynergistic models that would allow them to say that they had demonstrated synergism. Definitions would frequently be strained in order for the demonstration to be judged effective. Let us say that a valid demonstration of synergism might be one in which the combination of agent A (which is ineffective by itself) and agent B yields a greater effect than does agent B alone. Some investigators in using this approach equate treatment with an ineffective dose of an effective agent with treatment with an ineffective agent—these are two quite different things. In more extreme efforts to demonstrate synergism, some investigators treated agent A as being ineffective when the data showed some effect, albeit an insignificant one, for treatment A.

There is one interesting consideration that frequently attends demonstrations of synergism—"inexplicability." Apparently, some investigators think that a synergistic effect must be one that cannot be explained. With this interpretation, a demonstration of synergism would really be something negative (ie, we do not know what is happening) rather than something positive. Moreover, synergism would be something ephemeral: although we have no explanation for it today, we may have an explanation for it tomorrow. I would consider the question of inexplicability to be beside the point. If two agents yield an improved therapeutic effect when used in combination, I would take this as an indication of therapeutic synergism, and I would characterize it as explained or unexplained, as the case might be.

Dr. Eagle's example is interesting in this respect. He gives as a rationale for his synergistic combination an explanation in terms of resistance: If agent A fails in the treatment of syphilis it may be because some small number of treponema organisms, perhaps 0.1%, are resistant to the agent. Similarly, agent B may fail to cure syphilis because 0.1% of the organisms are resistant to that agent. If the resistance of the treponema organism to one agent is independent of resistance to a second agent, then when the two agents are given in combination the treponema-kill percentage would be 99.9999%; at that level of effectiveness, complete extinction of all of the cells in a limited infection would be a likely result and the infection would be cured. According to this explanation, the two agents are simply exerting their separate effects and are in no way interacting with each other. Yet, while treatment with each agent alone is incapable of producing cures, treatment with the two agents together does yield cures. After Dr. Eagle had published his results together with his rationale for the synergistic effect, he indicated to me that he would be unwilling to characterize the combined effect as a synergistic one, since he now viewed it as explainable in terms of the separate effects of each agent.

The concept of therapeutic synergism does not make life easy. It requires the expenditure of more effort in testing agents in more numerous and more imaginative ways. It also has a certain ephemeral quality: if I demonstrate A + B to be an especially good combination today, perhaps tomorrow someone will devise a new way of using A alone so that it is even better. However, for a while at least, the combination of drugs will have provided the therapy of choice.

How A and B are used together creates a new problem. They could be administered simultaneously, but perhaps an alternate-therapy schedule would be preferable. Note, however, that with the alternate-therapy schedule, treatment is given more frequently than when each agent is given alone. The remedy in this case would be to use two different kinds of schedules when the agents are tested alone: if the combination schedule is ABABAB . . . , the alternate A schedules would be A–A–A– . . . and AAAAAA. . . . (If for the preservation of times of treatment with B in the combination schedule the schedule for administration of B alone is –B–B–B . . . , there could be a loss in therapeutic effect due to the resulting delay in the initiation of treatment.) One justification for the use of an alternating schedule (not limited to the simple ABABAB . . . form) is that it keeps the environment for the pathogenic organism or tumor cell changing and, thus, the treatment could make the emergence of resistance through adaptation to the environment more difficult. When two agents are administered simultaneously, they can create only a single constant environment to which the organism would have to adapt.

Other desiderata govern the testing of drug combinations. For example, it would be pointless to test a combination for therapeutic synergism when one of the agents in the combination provides a near-perfect therapy for the experimental challenge used. Instead, the challenge should be made more extreme. Thus, if one of the agents in a combination is effective when used in the early stages of disease, combinations including this drug should be tested against advanced disease. Another way to increase the challenge might be to limit the method of treatment. If an agent is especially effective when given in multiple treatments, limit the treatment to single-day administration.

Statistical Designs for Experiments in Combination Therapy

Irwin Miller [1]

If an understanding of the nature of the dose-response curve is essential in determining the effect of a single compound, then it is even more critical in understanding the joint or combined effect of two or more treatments given sequentially or in combination.

This point is illustrated by the typical dose-response curve for a compound presumed to be active shown in figure 1. Knowledge of the full curve allows us to define (*a*) the region of activity, (*b*) the dose corresponding to maximum activity, and (*c*) the region of toxicity. Because random error is inevitable in all laboratory measurements, we are forced to recognize that we can never be certain of the active region. Thus, there are shaded areas in the figure. The term "response" will be used generically throughout this discussion to mean the particular measure of efficacy that is germane to the experiment at hand. In figure 1, we suppose that the response is the usual ratio of response in treated subjects to response in control subjects (T/C) given in percent and that the cutoff limits for activity and toxicity are close to those generally used with the L1210 leukemia system in screening.

If the dose-response curve is "flat" in the region of activity, different screeners will be reporting different optimum doses. This need not be a serious problem, however, because the *levels* of activity as measured by the response should be closely reproducible.

In the designing of experiments to measure the activity of a compound, the selection of doses that "cover" the active region (if any) is important. Exercising this caution is difficult enough in practice when only a single compound is used. It becomes even more difficult when we take into account the fact that the activity of some compounds is schedule dependent. Thus, we might find it necessary to consider a surface such as that illustrated in figure 2.

As is illustrated by the cross sections in this figure, there may be a different dose-response curve for each of several regimens. As is the case in this example, the compound may be less active for certain schedules and more active for others.

When two or more compounds (or other treatments, such as radiotherapy) are given in a combined regimen, we are always faced with the problem of defining a surface in at least three dimensions and possibly a "hypersurface" in many dimensions. Figure 3 illustrates such a surface for combination chemotherapy with two compounds in a fixed (but unspecified) regimen. Again, we can see that the response surface may be convoluted in such a way that little or no activity is apparent with increasing doses of one compound unless the dose level of the other compound is within a certain range.

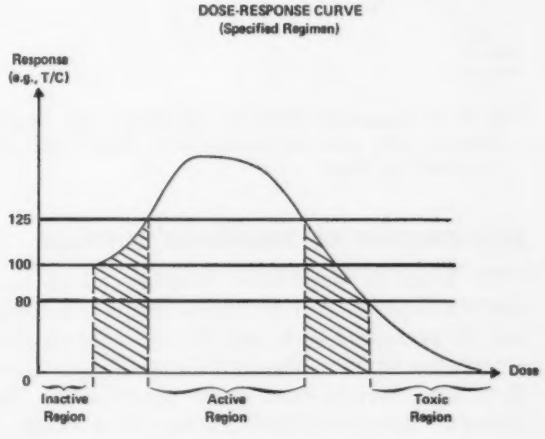

FIGURE 1.—Typical dose-response curve for a compound presumed to be active.

[1] Arthur D. Little, Inc., Cambridge, Mass.

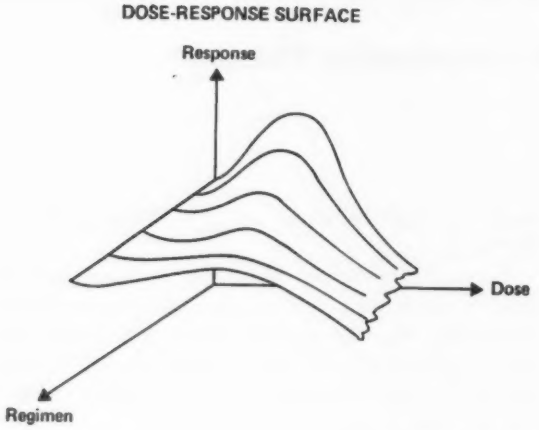

FIGURE 2.—Typical dose-response surface for single-agent chemotherapy.

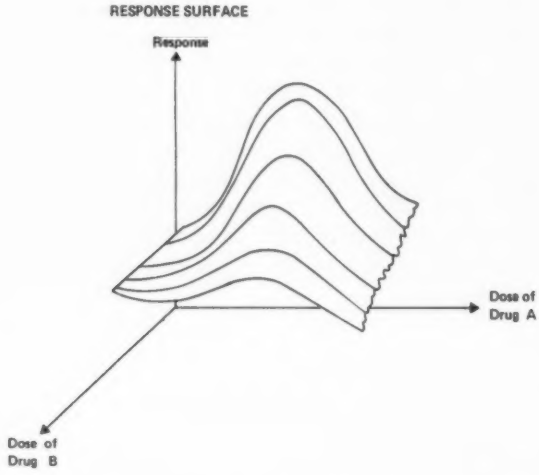

FIGURE 3.—Response surface for combination chemotherapy with two compounds in a fixed (but unspecified) regimen.

EXPLORATION OF RESPONSE SURFACE

In order to determine whether there is a combination of two or more compounds that can be presumed to be active, we must explore a response surface like that illustrated in figure 3, or one that is even more complicated. Because of the possibility of synergistic effect, it is not enough to explore this surface only over the range of doses for which the individual compounds have been shown to be active when used one at a time. Thus, for example, it is quite possible that the optimum doses for agents when they are given in combination chemotherapy may be quite different from the optimum doses for each compound when the compounds are given one at a time.

As the number of compounds (or other treatments, such as radiotherapy) to be given in combination increases, it becomes exponentially more difficult to adequately explore the response surface for signs of activity without conducting a large number of experiments. Fortunately, there are powerful statistical experimental designs that can drastically reduce the number of test groups required without sacrificing the ability of the experiment to locate the region of optimal activity. In fact, when judiciously and carefully applied, these statistical methods not only reduce the sample sizes required, they also increase the likelihood that an active region will be found if it exists.

Basically, these methods describe contours of equal activity, thereby drawing a "map" of the response surface similar to a topographic map of the surface of the earth. Figure 4 shows an example of such a contour map. The contours of equal activity, which I have dubbed "isoactives," are labeled with the value of the corresponding response (for example, T/C).[2] Note that this contour map shows a very high peak corresponding to presumed activity at a T/C level of 154%, a peak that could easily be missed even in the most intense series of experiments.

SOME EXPERIMENTAL DESIGNS

The most commonly used method of response-surface exploration involves experimental designs called "fully balanced factorial designs." Such factorial designs are generally divided into categories depending upon their ability to detect curvature in the response surface. The so-called *first-order designs* can detect only first-order (linear) effects (this in-

[2] After I had prepared this paper, Dr. Kensler (Arthur D. Little, Inc.) brought to my attention an article by S. E. deJongh entitled "Isoboles" (*In* Quantitative Methods in Pharmacology. Amsterdam, North Holland Publishing Co., 1961, pp 318–327). It appears that Dr. deJongh's "isoboles" are analogous to my "isoactives."

cludes simple interactions [synergies]). An example of such a design is shown in figure 5 where three compounds (effects) are given in combination and each compound is given at two dose levels. Note that all possible combinations of the two doses of each compound (eight combinations) are used. In order to measure reproducibility, it is usually necessary to repeat or "replicate" the entire experiment; therefore, 16 treatment groups would have to be used in this case.

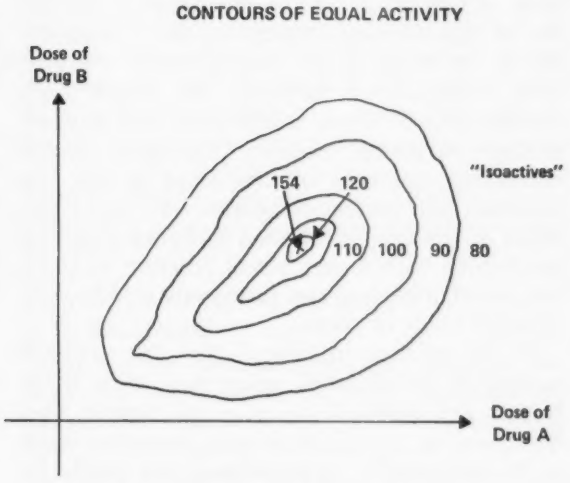

FIGURE 4.—Contours of equal activity.

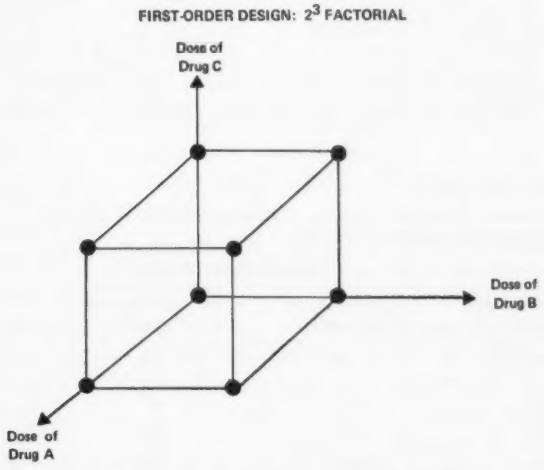

FIGURE 5.—First-order design: 2^3 factorial (three compounds or effects given in combination and each compound given at two dose levels).

Although fractional replication would not normally be recommended for an experiment with only three factors, it can be used to reduce the number of required test groups when a larger number of factors is to be used.

In figure 6, a carefully selected fraction of the full set of factorial treatments is chosen (in this case, one half of the full set). Again, some replication is necessary in order to measure "experimental error." When satisfactorily replicated fractional factorials are used, we lose some information about synergy; this is the price we pay for economy in the size of the experiment.

Most dose-response curves, as well as most response surfaces, are curved. Thus, they are not adequately represented by a single first-order experiment, and we are well advised to consider second-order designs. Unfortunately, fully balanced second-order factorial designs require very large numbers of test groups, especially as the number of factors increases. Special designs, which allow for sufficient replication to adequately measure experimental error and which can explore curved response surfaces, have been developed and applied by statisticians.

In figure 7, a second-order design for three factors is illustrated. This design is called a

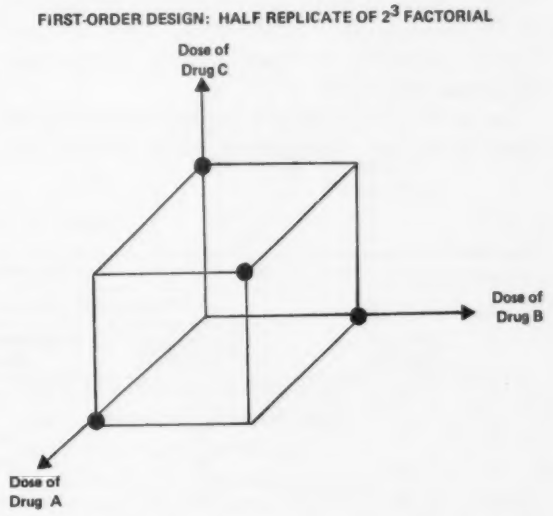

FIGURE 6.—First-order design: half replicate of 2^3 factorial.

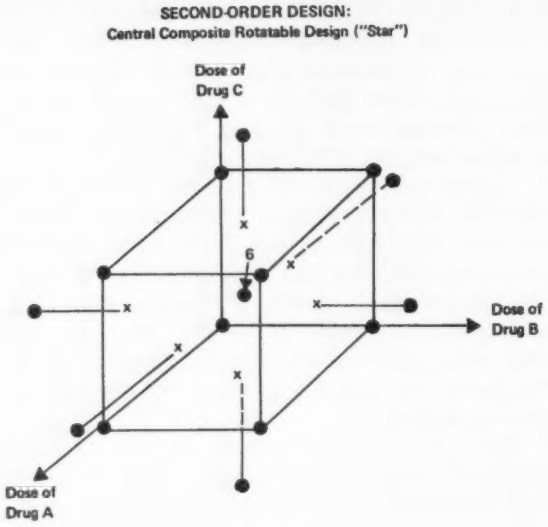

FIGURE 7.—Second-order design for three factors.

"central composite rotatable second-order design." Because of its name and structural appearance, it is often referred to as a "star." Note that it is sufficient for us to replicate only the center point (instead of going to the expense of replicating the entire design) to measure experimental error. In this case, we give six treatment groups the dosage combination represented by the center point in the "star." Such designs are extremely economical in response-surface exploration. They are also highly powerful in their ability to estimate optimum responses.

In table 1, some of the experimental designs that have been introduced on a purely conceptual level are summarized by a listing of the sample sizes that they require for combination chemotherapy experiments involving two through six factors. The replicated factorial designs—even the first-order designs—require many experimental groups, with the number required increasing very rapidly as the number of factors increases. Great economies may be achieved in first-order designs through the use of fractional factorials as is shown in the fourth column of the table. (The numbers given in parentheses in the third column of this table are the fractions of the full set of experimental treatments that are used.) When we come to the second-order designs, even unreplicated factorials (in which each combination of doses is given to only one experimental group) require large and rapidly increasing numbers of treatment groups. In contrast, the central composite, or "star," designs of the type illustrated in figure 7 can be performed with a very small fraction of these treatment groups when the number of factors is large (four or more).

It can be seen in table 1 that the greatest economies are obtained when more than three factors are involved, and it would appear that little can be gained when only two-compound or two-treatment combinations are used. In fact, many experiments that appear to be two-compound experiments really include other factors relating to choice of regimen. In addition, in the examination of only two treatments with each given at many dose levels, it is often a useful statistical procedure to "pseudofactor," that is, to break up the dose levels of a single

TABLE 1.—Size of experiment

No. of factors	No. of experimental groups required			
	First-order designs		Second-order designs	
	Replicated 2^k factorial	Replicated fractional factorial*	Unreplicated factorial	Central composite ("star")
2	8	(1) 8	9	13
3	16	(1)16	27	20
4	32	(½)16	81	31
5	64	(¼)16	243	32
6	128	(¼)32	729	53

*Numbers in parentheses are the fractions of the full set of experimental treatments that are used.

compound into sets of two levels each and regard these sets as separate "factors" for purposes of experimental design. Thus, four-, five-, and even six-factor experiments come into play in statistical design, even when only two compounds are tested.

The purpose of this rather quick and "dirty" review of some experimental designs for response-surface exploration has been to emphasize their latent economic advantages. This is probably neither the time nor the place for the mathematical details of how the designs are set up or how they are analyzed. It is sufficient to say, however, (*a*) that the setups are not as complicated as they might appear at first, (*b*) that it would be a simple matter to provide instructions to the screening laboratories on what experimental groups to use, and (*c*) that the statistical analyses of their results are easily performed by trained analysts and are quite easily programmed for computer use.

LOCATING OPTIMUM DOSAGE COMBINATIONS

So far, we have discussed experimental designs for combination chemotherapy with the general purpose of exploring the dose-response surface and describing its properties. In presumptive screening, however, we are not necessarily concerned with the entire response surface; we are more interested in determining the dosage combinations corresponding to optimum response and in determining whether this optimum response is large enough for us to presume that the combinations are active.

For this more restricted purpose we can use methods designed to locate optimum values on response surfaces quickly and with a minimum of excess experimentation. One such method is the method of *steepest ascent* first proposed by G. E. P. Box and K. B. Wilson in connection with multifactor chemical experiments in industry. This method is in wide use today, and there is an impressive body of literature describing the method and results of its application. It is especially applicable to primary screening, in my opinion, because it requires a limited number of test groups and because it proceeds in a series of very simple steps.

As is described in figure 8, we first do a replicated first-order design, centering this design in the region where we might initially expect the greatest amount of activity. Then, using simple mathematical methods (methods for which easy-to-use nomographs could be developed), we calculate the "path of steepest ascent." This path is the one that "gets us up the hill" the fastest. We then choose a point on this path that is far enough from the original experimental set to be meaningful (but not so far that we run the risk of "going over the top"), centering a new first-order design at that point. It is often easy to tell just by looking at the data when we have "straddled" the maximum response, and statistical methods that are easy to program can be used to estimate the optimum response. If for some reason we should later want a more accurate estimate of the optimum response, we can then center a "star" design on this estimate. We could base our judgment of presumed activity for the combination chemotherapy regimen on this more accurate estimate of optimum response.

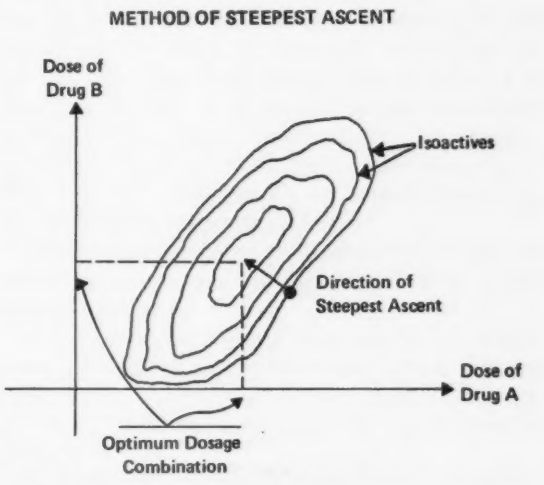
METHOD OF STEEPEST ASCENT
Dose of Drug B
Isoactives
Direction of Steepest Ascent
Dose of Drug A
Optimum Dosage Combination
Step 1. Do a replicated first-order design
Step 2. Calculate path of steepest ascent
Step 3. Test one group on this path
Step 4. Do new first-order design centered on new point —
Keep going until plateau of optimum activity is reached —

FIGURE 8.—Method of steepest ascent.

SUMMARY

Having attended a number of these screeners meetings, I have learned a great deal, but I have felt a little bit like a biologist at a statistical convention. I am happy to have had the opportunity to turn the tables today. In a more serious vein, I have attempted to convey to you the concept that there already exists a variety of tested—tried and true—statistical methods for experimental design that I believe will be useful as your program of combination chemotherapy develops and progresses. Clearly, much work needs to be done to apply these methods and get the most out of them. I strongly believe that the economies and power of the method will do far more than merely justify this work.

Combination Chemotherapy: Considerations for Design and Analysis [1]

Harris H. Lloyd [2,3]

The objective of combination chemotherapy is the improvement of a specified response by the administration or application of two or more chemical agents. Normally, improvement of a response infers, qualitatively, that treatment by a combination of drugs provides a "better outcome" than could be achieved by the use of any of the drugs alone. As applied to the treatment of disseminated cancer in experimental animals or man, the objective of combination chemotherapy is the achievement of greater tumor cell kill with acceptable host toxicity and greater cure rates than can be obtained by treatment with single drugs.

Selecting drug combinations, optimizing schedules and doses, and demonstrating quantitatively that the combination treatment improves response relative to the corresponding single-drug treatments involves considerable effort. The demonstrated usefulness that combination chemotherapy has had in treating cancer in experimental animals and man would suggest that new drug combinations could lead to continuing improvement in therapeutic response.

The purpose of this report is to review some of the factors that should be considered in selecting drug combinations and quantitatively assessing their usefulness. It is not intended to be comprehensive nor even complete within its scope. For instance, other modalities of treatment such as surgery, radiation therapy, and immunotherapy are not considered; and only selected topics of experimental design and analysis are discussed. Included is a brief discussion of computer-based information management.

CHOOSING DRUG COMBINATIONS

Many drug combinations have been found and evaluated by trial and error. A trial and error selection or random combination of drugs, though demonstrating some usefulness, must eventually lead to difficulties. For instance, in 1972 the National Cancer Institute reported that there were 67 drugs either clinically active or of current interest.[4] This number seems certain to increase. Consider the number of possible combinations of two or more drugs that could be derived from 67 drugs (table 1). The figures in table 1 indicate that it would not be feasible to attempt a thorough evaluation of all the possible drug combinations, even for this limited set of drugs. It seems necessary to select those drug combinations that are potentially effective or, at least, eliminate those that are not likely to be an improvement on single-drug treatments.

Some considerations that could be helpful in selecting drug combinations are briefly summarized below.

1. In vivo single-drug experience: It would seem preferable to consider only those agents that have objectively demonstrated activity against established tumors in experimental animals or man.

2. Cell culture experience: Drug combinations demonstrating potentiating cytotoxicity against established tumor cell lines in vitro could suggest further testing in animals.

3. Combination toxicity: Drug combinations that are less than additive in host toxicity are

[1] Supported by contract NIH-71-2098 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Mathematical Biology and Data Analysis Section, Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, Ala.

[3] The author thanks those associates who have patiently taught him biology, particularly Drs. Frank M. Schabel, Jr., Howard E. Skipper, Lee J. Wilkoff, and Linda Simpson-Herren. Discussions with these and many other members of the biologic sciences group at Southern Research Institute have helped to formulate this paper.

[4] Report of the Chemotherapy Program, NCI. Bethesda, Md, National Cancer Institute, vol 2, 1972.

TABLE 1.—Number of drug combinations available from 67 individual drugs

No. of drugs in combination	No. of combinations
2	2211
3	47,905
4	766,490
5	$\sim 10 \times 10^6$
6	$\sim 100 \times 10^6$
7	$\sim 870 \times 10^6$
8	$\sim 6.5 \times 10^9$

frequently potentiating in their effect against tumors in experimental animals and man.[5]

4. Cross resistance: Drugs in combination that are not cross resistant toward the treated cell population will be more effective than those drugs that do exhibit cross resistance (1).

5. Biochemical mechanisms: Knowledge of the nature of activation or site of action could be useful in selecting potentiating drug combinations or eliminating those that may not be potentiating.

6. Chemical, physical, and biologic properties: Classifying drugs by chemical activity (eg, alkylation, consideration of solubility or partition coefficients) and grouping drugs according to cytotoxicity patterns (eg, cell-cycle specific, cell-cycle-stage specific, etc) could be useful in selecting combinations of drugs that would be potentiating.

CHOOSING SCHEDULES AND DOSES

Optimal schedules and doses for many drugs have been determined for the treatment of L1210 leukemia (2). Principles underlying schedule dependency and a rationale for applying the results observed in the treatment of one tumor system toward estimating the optimal schedule in another have been described (3). It appears that the extrapolation of an optimal schedule from one tumor system to another can be accomplished with only quantitative changes, eg, changes in interval between doses or number of courses. It might therefore be expected that drugs in combination could best be administered (at least until subsequent testing indicated otherwise) by the optimal schedules either observed or predicted for the use of each drug individually. The drugs in combination may be applied *simultaneously*, at *staggered* intervals, or *sequentially*. Simultaneous scheduling means each drug is administered on an identical schedule, each dose being given at the same time. Staggered scheduling means each drug is administered on an identical schedule but displaced in time such that the dose of one drug follows that of another at a constant interval. Sequential scheduling means that all of the doses of one drug are administered according to schedule before a subsequent drug is administered.

The selection of doses for the individual drugs in a combination poses a different problem. Methods that may be applicable to the determination of optimal doses will be presented later. Table 2 gives the possible dose combinations for two drugs each administered at three dose levels. There are a total of nine dose combinations. If each drug should require individual testing at the same dose levels, six more tests would be necessary. The doses used in combination are expressed in terms of the fraction of LD10 when the drug is administered individually according to the schedule used in the combination. In table 2 the dose combinations are grouped according to the sum of the fractions of the individual LD10s. It has been suggested that each group represents, at least approximately, dose combinations that are equally toxic even though the proportion of the individual drugs within the combination may vary (1). The therapeutic response within each group may depend upon the proportion of each drug present in the combination.

TABLE 2.—Possible dose combinations for two drugs, each tested at three levels: ⅓ LD10, ⅔ LD10, and 1 LD10

	A	B	A	B	A	B	A	B	A	B
					1/3	1				
			1/3	2/3			2/3	1		
	1/3	1/3			2/3	2/3			1	1
			2/3	1/3			1	2/3		
					1	1/3				
$\sum LD_{10}$	2/3		1		4/3		5/3		2	
N	1		2		3		2		1	

[5] Skipper HE. Unpublished observations.

TABLE 3.—Possible dose combinations for three drugs, each tested at three levels: ⅓ LD10, ⅔ LD10, and 1 LD10

A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
									1/3	2/3	1						
						1/3	1/3	1				1/3	1	1			
									1/3	1	2/3						
						1/3	2/3	2/3				2/3	2/3	1			
			1/3	1/3	2/3				2/3	1/3	1				2/3	1	1
						1/3	1	1/3				2/3	1	2/3			
		1/3	1/3	2/3	1/3				2/3	2/3	2/3				1	2/3	1
						2/3	1/3	2/3				1	1/3	1			
			2/3	1/3	1/3				2/3	1	1/3				1	1	2/3
						2/3	2/3	1/3				1	2/3	2/3			
									1	1/3	2/3						
						1	1/3	1/3				1	1	1/3			
									1	2/3	1/3						
$\sum LD_{10}$		1		4/3			5/3			2			1/3			8/3			3	
N		1		3			6			7			6			3			1	

It would certainly be feasible to test all of the dose combinations listed in table 2, but a limited set of combinations could also be chosen for testing. For instance, a group with approximately equal toxicity could be selected to test the dependence of dose ratio on therapeutic response while simultaneously, at least partially, eliminating complications due to varying host toxicity. Or perhaps one dose combination from each group could be selected to obtain a total dose response. Table 3 lists all the dose combinations, grouped according to toxicity, for three drugs tested at each of three levels and illustrates further the desirability of restricting the test space as the number of drugs used in combination increases. Further restriction of the test space may be required if more than three dose levels are deemed necessary for adequate evaluation. Also, if it is not known that the three-drug combination (A + B + C) is in fact an improvement over one of the possible two-drug combinations (A + B, A + C, or B + C), then each of the two-drug combinations should also be evaluated. A dose response for each of the individual drugs may also be required. Therefore, a complete evalua-

TABLE 4.—Possible total dose combinations expressed as the sum of single-dose LD10s for up to six drugs in combination, with each drug tested at three equally spaced levels

INDIVIDUAL DOSES (FRACTION OF LD_{10})		$\sum LD_{10}$																					
1/3, 2/3, 1		2/3	1	4/3	5/3	2	7/3	8/3	3	10/3	11/3	4	13/3	14/3	5	16/3	17/3	6					
1/4, 1/2, 3/4		1/2	3/4	1	5/4	3/2	7/4	2	9/4	5/2	11/4	3	13/4	7/2	15/4	4	17/4	9/2					
1/6, 1/3, 1/2		1/3	1/2	2/3	5/6	1	7/6	4/3	3/2	5/3	11/6	2	13/6	7/3	5/2	8/3	17/6	3					
NO. OF DRUGS	TOTAL COMB.																						
2	9	1	2	3	2	1																	
3	27		1	3	6	7	6	3	1														
4	81			1	4	10	16	19	16	10	4	1											
5	243				1	5	15	30	45	51	45	30	15	5	1								
6	729					1	6	21	50	90	126	141	126	90	50	21	6	1					

tion of a three-drug combination would require 66 tests.

Table 4 is a summarized generalization of tables 2 and 3 and contains information for combinations of up to six drugs. The total number of combinations is shown when each drug is used at three equally spaced dose levels. Also, the number of combinations which would exhibit approximately equal host toxicity can be found for three sets of test doses. This is to illustrate how a range of host toxicities may be chosen or how excessive toxicity may be avoided. The sum of the fractions of the individual LD10s does not necessarily imply the observed toxicity of the combination will equal the sum. The actual toxicity for the combination will require experimental determination. Table 5 is similar to table 4, except that testing of the combinations is assumed for two equally spaced doses instead of three. The number of possible dose combinations and the number of "equitoxic" combinations is less for two dose levels than for three dose levels.

CHOOSING THE SIZE OF TEST AND CONTROL GROUPS

Questions regarding sample size are frequently asked. Specifically, an investigator planning therapeutic trials of drugs or drug combinations in an experimental tumor system would desire to know the number of animals required to achieve statistical significance for a comparison between test and control groups. Only an estimate of the required number may be given, and this estimate must be based on certain assumptions and at least a guess about the magnitude of the variances of the control and test distribution. The initial estimate of the sample size may require adjustment if so indicated by the data collected.

The "student's t" test will be used to obtain an equation for sample size. Hence,

$$t = \frac{\left|\bar{x}_t - \bar{x}_c\right|}{\sqrt{S^2\left(\frac{1}{n_t} - \frac{1}{n_c}\right)}} \qquad (1)$$

where $\bar{x}_t$ and $\bar{x}_c$ are the means for the test and control groups respectively, n_t and n_c are the number of animals in the test and control groups, and S^2 is given by

$$S^2 = \frac{(n_c-1)\sigma_c^2 + (n_t-1)\sigma_t^2}{n_c + n_t - 2} \qquad (2)$$

where σ_c^2 and σ_t^2 are the variances of the two populations. Assume that the variances and sample sizes of each group are related by

$$\sigma_t^2 = k \cdot \sigma_c^2 \qquad (3)$$

$$n_c = a \cdot n_t$$

where k and a are constants and may have any positive value. Substituting equation (3) into (2) gives

$$S^2 = \sigma_c^2 \frac{(a + k)n_t - (k + 1)}{(a + 1)n_t - 2}$$

which may be rearranged to

$$S^2 = \frac{\sigma_c^2}{n_t}\left\{\frac{k}{a}\left(\frac{n_t - 1}{n_t - \frac{2}{a+1}}\right) + \left(\frac{n_t - \frac{1}{a}}{n_t - \frac{2}{a+1}}\right)\right\}.$$

TABLE 5.—Possible total dose combinations expressed as sum of single-dose LD10s for up to six drugs in combination, with each drug tested at two equally spaced levels

INDIVIDUAL DOSES (FRACTION OF LD_{10})		$\sum LD_{10}$											
1/2, 1		1	3/2	2	5/2	3	7/2	4	9/2	5	11/2	6
1/3, 2/3		2/3	1	4/3	5/3	2	7/3	8/3	3	10/3	11/3	4
1/4, 1/2		1/2	3/4	1	5/4	3/2	7/4	2	9/4	5/2	11/4	3
1/6, 1/3		1/3	1/2	2/3	5/6	1	7/6	4/3	3/2	5/3	11/6	2
NO. OF DRUGS	TOTAL COMB.											
2	4	1	2	1								
3	8		1	3	3	1						
4	16			1	4	6	4	1				
5	32				1	5	10	10	5	1		
6	64					1	6	15	20	15	6	1

The expressions in parentheses are approximately unity. This is a good approximation for large n_t but there will be some error when both n_t and a are small. Therefore,

$$S^2 \simeq \frac{\sigma_c^2}{n_t} \left(1 + \frac{k}{a}\right). \quad (4)$$

Let $|\delta|$ represent the minimal or desired difference to be detected between the test and control groups. Equation (1) now becomes

$$t \simeq \frac{|\delta|}{\sigma_c \sqrt{\frac{1}{n_t}\left(1 + \frac{k}{a}\right)}}.$$

Using the value of t for 95% confidence and rearranging gives

$$t_{95} \sqrt{\frac{1}{n_t}\left(1 + \frac{k}{a}\right)} \simeq \frac{|\delta|}{\sigma_c}. \quad (5)$$

Typically, the ranges of k and a are $4 \leq k \leq 10$, and $1 \leq a \leq 30$, which means that $0.13 \leq \frac{k}{a} \leq 10$. Table 6 lists the values of $|\delta|/\sigma_c$ for a range of $\frac{k}{a}$. The estimate of n_t may be found in the left-hand column reading horizontally from the appropriate value of $|\delta|/\sigma_c$. Therefore, with this sample size we can expect to detect differences that are equal to or greater than $|\delta|$. The table may be expanded to include intermediate values of n_t not listed.

Only a value for n_t is given in table 6 because of the assumed known relationship between n_t and n_c. If concurrent controls are included with the test group, then typically $n_c \simeq n_t$ and $a \simeq 1$. If the test group is compared to a historical or cumulative control, then $n_c >> n_t$ and $a >> 1$.

EVALUATION OF THERAPEUTIC RESPONSE

The results of an experimental therapeutic trial may be expressed in several ways. It is not always possible to adequately express the outcome of an experiment as one number, but some attempt at data reduction is justifiable. The list of variables below has been applied to single-drug therapeutic trials as well as to combination chemotherapy.

1. Survivors.—The number of survivors in the test group or the percent of survivors on some day of evaluation is a good indicator of treatment efficacy. Long-term survivors suggest "cures." In experimental tumor systems where the disease can recur by induction (AKR leukemia, for instance) and for treatments that exhibit delayed toxicity, long-term survivors may not occur even though "cures" are achieved.

2. Increase in lifespan.—The increase in lifespan, or the percent of increase in lifespan at the median lifespan of the control and treated groups, is also used as a measure of treatment effectiveness. This parameter is relatively easy to compute and is not affected greatly by animals that die early due to unusual host sensitivity to the treatment or by a few animals that survive for a considerable period. The increase in lifespan may be computed at other points along the survival curve such as the 75% or 25% survival. The average difference or percent of increase in lifespan may also be computed, but this quantity is influenced to a greater extent than the median by extremely early or late deaths. Whenever the test group has survivors, these animals should not be included in the computation of the median or mean. The result of the experiment could then be expressed by the increase in lifespan of the dying animals and the number of survivors.

3. Survival after end of treatment.—The mean or median duration of survival after the end of treatment can be related to the cell or tumor burden at the end of treatment. In general, the duration of survival is inversely proportional to the tumor burden at the end of therapy.

4. Tumor weight or cell number.—The number of cells or viable tumor surviving treatment is a direct measure of the effectiveness of therapy. This quantity is frequently expressed as "fraction surviving" or "log kill" relative to some standard such as tumor burden at initiation of treatment. The use of lifespan as a quantitative indicator of cell burden has been well established for the L1210 leukemia system (4). This technique is a sort of in situ bioassay and works well for exponentially growing cell populations or for populations whose growth

TABLE 6.—To estimate the number of animals required to detect a difference between the control and test groups of $\geq |\delta|$ at the 95% confidence level; estimate σ_c, k, and a (if not known) and find the appropriate $|\delta|/\sigma_c$ in the table

	$\frac{\lvert\delta\rvert}{\sigma_c}$								
K/A^* = N_T	~0	0.5	1	2	3	4	6	8	10
5	1.15	1.41	1.62	1.99	2.30	2.57	3.04	3.45	3.81
10	0.70	0.86	1.00	1.22	1.41	1.58	1.86	2.11	2.34
20	0.47	0.57	0.66	0.81	0.93	1.04	1.23	1.40	1.55
30	0.37	0.46	0.53	0.65	0.75	0.83	0.99	1.12	1.24
50	0.28	0.35	0.40	0.50	0.57	0.63	0.75	0.85	0.94
100	0.20	0.24	0.28	0.34	0.39	0.44	0.52	0.59	0.65
500	0.09	0.11	0.12	0.15	0.18	0.20	0.23	0.26	0.29

* $\sigma_T^2 = K\sigma_C^2$ AND $N_C = A \cdot N_T$

approximates exponential. For these cases it may be shown that

$$N_S = N_O 2^{\{b-(T-C)\}/\delta} \qquad (6)$$

where N_S is the number of cells surviving at time b (the time of the last treatment relative to inoculation). N_O is the size of the inoculum at time zero which may be corrected for viability, T and C are the mean or median lifespan of the treated and control groups respectively, and δ is the exponential doubling time of the cell population. When the fraction surviving (f_S) is related to the initial inoculum, then

$$f_S = \frac{N_S}{N_O} = 2^{\{b-(T-C)\}/\delta}$$

and it may be obtained without knowing the exact magnitude of N_O provided the inoculum is identical for both treated and control groups. The derivation of equation (6) presumes some constant cell number that causes host lethality. The log kill is defined as

$$\text{log kill} = -\log_{10}(f_S). \qquad (7)$$

This analysis can be extended to include the computation of cell kill per course or per dose for multiple-dose therapy.[6]

In general, solid tumors do not grow exponentially, but their growth may be described satisfactorily by a Gompertz function (5). Determining tumor size by caliper measurements following therapy usually underestimates substantially the effectiveness of treatment (6), probably due to the time delay required to remove dead tumor cells and the relatively rapid repopulation resulting from the surviving tumor cells. This phenomenon has been substantiated by bioassay of solid tumors following treatment. However, if the Gompertz function is known for the experimental tumor system and if it is assumed that the treated tumor begins to repopulate immediately following therapy obeying the same Gompertz function as untreated controls, then it may be shown[7] that

$$\text{log kill} = \frac{\beta}{\alpha} e^{-\alpha T} \left(e^{\alpha(\epsilon-d)} - 1\right) \qquad (8)$$

where α and β are the Gompertz parameters, T is the time relative to time zero that treatment was started, d is the time difference between the first and last treatments, and ϵ is the displacement in time between the control and treated Gompertz curves. ϵ may also be described as the time required for the tumor

[6] Southern Research Institute. Unpublished analysis.
[7] Lloyd HH. Unpublished analysis.

to regrow to its size at the time treatment was begun. Equation (8) is an estimate of the log kill relative to the tumor size at the time treatment was started, and is valid for both single-dose therapy ($d = 0$) and multiple-dose therapy ($d > 0$).

5. Survival curves.—Control and treated survival curves may be tested directly for significant difference (7). Such a test could be performed in addition to or in lieu of the evaluation of any of the parameters listed above.

6. Dose levels for specified response.—For studies with single drugs the determination of quantities such as LD10, ED50, ED99, etc, is straightforward and only requires measuring the specified biologic response at a series of dose levels followed by an analysis (such as probit analysis for determining LD10) which estimates the required dose from the collected data. For treatment with more than one drug such an evaluation is not so straightforward. The specified dose level must be expressed for one of the drugs while the other drugs are held at a constant level. Additionally, the dose combination which provides the optimum response could be determined.

FINDING THE "BEST" DOSE COMBINATION

The "best" or optimum dose combination may be defined as the dose levels of the drugs in a combination that provide maximum therapeutic effectiveness without exceeding an acceptable host toxicity. For the purposes of this discussion, it will be presumed that the optimum dose combination refers to some stated schedule for the administration of each drug in the combination. The optimum dose combination will most probably change with the schedule, and a general analysis would also consider schedule dependency in the optimizing technique. The dose combination just causing acceptable host toxicity without exceeding it is referred to as the maximum tolerated dose (MTD) and in experimental systems it is seen as equivalent to the LD10. Therefore, the optimum dose combination will not exceed the MTD. Therapeutic effectiveness may be quantitatively ascertained by measuring any of the parameters described in the previous section.

It is to be noted that the optimum dose combination does not necessarily mean that the drug combination is either potentiating or exhibits a greater-than-additive effect. A drug combination that is antagonistic would not be of any benefit. However, it is frequently of interest to determine the degree of interaction of the components of a drug combination as measured by some specified response. This may be done in cell culture systems or in vivo and the results are frequently presented as isobolograms (8). Figure 1 depicts the interpretation of isobolograms and illustrates two hypothetical examples for two-drug combination chemotherapy. It is first necessary to determine the dose of each drug that is required to elicit some specified response. Then dose combinations that elicit the same response are determined by measuring the response at a series of doses of one of the drugs while holding the other drug at a constant dose. The dose combinations are plotted as a fraction of the dose of the individual drug which produced the same response. Connecting the points with a smooth curve forms what is known as an "isobole." If the isobole (fig 1) lies within triangle AOC the drug combination is potentiating, if it lies along line AC it is additive, if it lies within triangle ABC it is greater than additive, and if it is outside ABC it is antagonistic. The right-hand side of figure 1 illustrates two hypothetical isoboles, one of which indicates a potentiating combination and one of which shows a combination that may be either antagonistic or greater than additive. In either case the optimum dose combination in the sense of providing the maximum response within the MTD has not necessarily been found. It is possible that a drug combination exists that will elicit a greater degree of response without

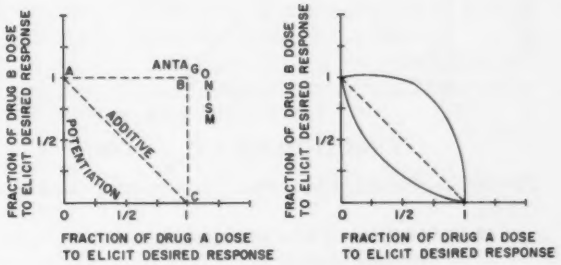

FIGURE 1.—Interpretation of isobolograms.

exceeding the MTD. This method of analysis becomes difficult for drug combinations of more than two drugs.

Figure 2 illustrates a method for finding the optimum dose combination for a two-drug combination (9). The axes are in units of the fraction of the LD10 of each individual drug in the combination. This choice of units is arbitrary, though convenient, and any other useful unit to express the dose is equally valid for the analysis. If it is assumed, as stated earlier, that the sum of the fraction of the LD10s of the individual drugs used in combination determines the toxicity, at least approximately, then the broken lines represent "isotoxic" dose combinations. The dots correspond to the dose combinations listed in table 2, although more dose combinations are feasible, and in practice the drugs would be tested individually, which would correspond to points along each axis. The solid lines proceeding from the origin represent dose combinations in which the dose ratio of the two drugs is constant. Suppose that some response is measured for each of the nine dose combinations shown by the data and also for each of the individual drugs tested at, say, three doses. If any of the doses or dose combinations exceeds the MTD, then it should be omitted from further analysis. The object of the analysis is to find the dose combination that provides the maximum response within the MTD. This could be done by utilizing some mathematical model (9) of the response surface, fitting the model to the observed points, and computing the maximum. Another analysis not requiring the formulation of an arbitrary mathematical model is known as the method of steepest ascent. If the maximum response lies near or on one of the axes, then the combination may be considered of no greater benefit than the most effective drug used alone. In principle, analysis by the method of steepest ascent may be extended to include combinations of more than two drugs and additional factors such as schedule. However, as stated previously, the number of possible dose combinations increases rapidly as the number of drugs used in combination increases and, therefore, methods need to be developed that will provide a means of selecting the minimum number of dose combinations to provide adequate response data for the analysis to succeed in finding the maximum response.

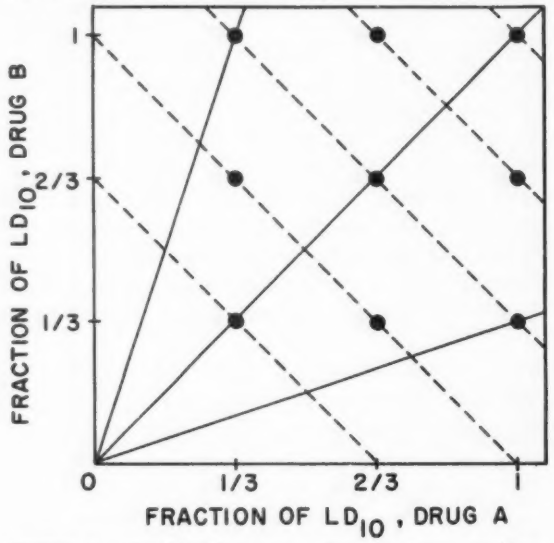

FIGURE 2.—Combination space for two-drug combination.
● = Selected test dose combination.
- - - - = "Isotoxic" line.
—— = Constant dose-ratio line.

COMPUTER-BASED INFORMATION MANAGEMENT

In addition to computer-assisted analysis of individual experiments, it is sometimes desirable, or even necessary, to maintain a computer-based management and retrieval system consisting of the data collected from a large number of similarly executed experiments. Figure 3 lists some of the most important considerations for planning and designing a computer-based information management system. The importance of the first two elements of figure 3 cannot be overemphasized, since the program details will be dependent upon the required performance of the system and the data base to be managed. A major change in either the system objectives or the content of the data base during the programming phase of system development can potentially lead to rewriting or scrapping all programs. Some flexibility of the data base content is feasible, but the major features of content should be established before programming begins. The format and structure of the stored data is dependent upon system

1. Establish objectives
2. Select data to be included in data base
3. Design data structures
4. Plan for unexpected additions or changes
5. Promote communication among users, designers, and programmers
6. Select mode of input
7. Design reports to be generated
8. Prepare programs
9. Establish plan for program and system maintenance

FIGURE 3.—Considerations for computer-based information management.

requirements, data storage devices available, programming language chosen, and available software. Frequent and open communication among system users, programmers, and analysts can lead to some flexibility in design such that potential and unexpected future changes or additions to the system can be anticipated. If the design is sufficiently flexible, changes and additions can be incorporated during regular maintenance. The degree of flexibility within the framework of established system objectives and data base content is limited, and should either of these change fundamentally for any reason then the best course of action would be to design a new system.

CONCLUDING REMARKS

It has not been the purpose of this report to dictate the details of experimental design for combination chemotherapy, but only to discuss some of the factors that should be considered for design. Among these are choosing drug combinations, schedules, dose combinations, and size of test and control groups; evaluating therapeutic response; establishing methods for determining dose combinations that provide maximum therapeutic response; and designing computer-based information management systems. The use or rejection of any of these considerations for experimental design is dependent upon the particular question to be answered and the resources available. Other factors not considered in this report are selection of route of administration and choice of experimental tumor and host. Neither of these are trivial choices, but again, they are dependent upon particular experimental requirements.

REFERENCES

1. SKIPPER HE, HUTCHISON DJ, SCHABEL FM, JR, ET AL. A quick reference chart on cross resistance between anticancer agents. Cancer Chemother Rep 56:493–498, 1972.
2. VENDITTI JM. Treatment schedule dependency of experimentally active antileukemic (L1210) drugs. Cancer Chemother Rep Part 3, vol 2:35–59, 1971.
3. SKIPPER HE, SCHABEL FM, JR, MELLETT LB, ET AL. Implications of biochemical, cytokinetic, pharmacologic, and toxicologic relationships in the design of optimal therapeutic schedules. Cancer Chemother Rep 54:431–450, 1970.
4. SKIPPER HE, SCHABEL FM, JR, and WILCOX WS. Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with "curability" of experimental leukemia. Cancer Chemother Rep 35:1–111, 1964.
5. SIMPSON-HERREN L, and LLOYD HH. Kinetic parameters and growth curves for experimental tumor systems. Cancer Chemother Rep 54:143–174, 1970.
6. WILCOX WS, GRISWOLD DP, JR, LASTER WR, JR, ET AL. Experimental evaluation of potential anticancer agents. XVII. Kinetics of growth and regression after treatment of certain solid tumors. Cancer Chemother Rep 47:27–39, 1965.
7. FORSYTHE AF, and FREY HS. Tests of significance from survival data. Comput Biomed Res 3:124–132, 1970.
8. HEWLETT PS. Measurement of the potencies of drug mixtures. Biometrics 25:477–487, 1969.
9. ADDELMAN S, GAYLOR DW, and BOHRER RE. Sequences of combination chemotherapy experiments. Biometrics 22:730–746, 1966.

Collection and Presentation of Combination Data

Rhoda R. Mancher [1]

Combination therapy has assumed an important role in the animal screen, and the data processing area of Drug Research and Development has felt the impact of this change of direction. During the past year, the number of combination chemotherapy control packs received has risen dramatically. In the 9 months from July 1972 through March 1973, 233 control packs containing a total of 7679 combination therapy tests were received in encoded format for data processing and storage on the files at the National Institutes of Health (NIH). By the end of March 1973, 8114 tests had been processed and stored and another 8282 tests had been logged in and were waiting for data processing. Many of these tests represent a backlog of data (stored in prior years at the screening laboratories) that is being moved onto the main files for future reference.

The tape files that hold the screening data have grown during the past 15 years to more than 3 million records. They were originally organized and still exist with the data grouped by NSC number. In such an environment, there is no way to store data for experiments in which two or more therapies are combined. Reorganization of the files would include extensive analysis, drastic change, great expense, and probably some interruption in service. With the funds and staff available, it was barely possible to keep the system going and progressing in the area of single-drug testing. Data-capturing forms were designed to support the protocol for the primary screen. They did not have the flexibility necessary for the variations that occur in any secondary testing and particularly in combination testing.

The first stage of the attack was to delineate the functions that such a system would have to accommodate with emphasis on data capture, storage, and reporting. Our immediate decision was to create a separate file which would be organized differently from the main files and which would be used for storing the raw data of the experiments rather than the results. On this new file, the data would be grouped by screener number, tumor designation, and control number. Thus, the NSC number became only one of a number of fields allowed to vary throughout these control packs. Although data could be retrieved by NSC number (which could represent either a synthetic or natural product or an alternate therapy such as radiation), it could also be retrieved by any other variable or constant field on the file.

In order to incorporate the data into the system, a scheme had to be devised whereby the control pack would have descriptors attached to it spelling out the variables in each test. Extensive analysis was required to develop criteria for which particulars, in a mainly empirical system where the number of variables allowed could reach very large proportions, would be most likely to vary within a control pack. Rules were then written for how control packs could and could not be set up. This was in some ways arbitrary, and experience later proved that in certain areas it would be necessary to change our specifications.

This system is still in the developmental stage. Thus, it is constantly being modified and expanded. As of now, the fields that are not allowed to vary within a control pack are first the primary descriptors (screener-tumor-control number) and then parameter (method of evaluation), inoculum tissue, tumor subline designations (tumor line, tumor code, and generation), sex and source of host, final evaluation day, initial weighing day, and toxicity evaluation day.

The fields that can vary are host, inoculum site, inoculum level, NSC number, vehicle,

[1] Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

route, regimen, day of first injection, number of injections, and dose level.

COLLECTING THE DATA

Once the system rules had been established, a method for collecting this highly variable data had to be devised. Consideration was given to versatility, ease of completion, and ease of collection. When the data is complex but straightforward presentation to the computer is required and when the persons completing the forms are widely dispersed, it is no simple matter to design a usable method of data collection. The first modification (later carried over to the primary screening data forms) was to put all of the information that was to remain constant throughout the control pack onto the Screening Control Record (*see* fig 1, form NIH-1744-1) with the control animal information. Some of the fields that would normally be encoded were made large enough to accept natural language since we wanted to capture as much of the data as possible (and avoid "other" codes). However, as we began to gain experience in completing the forms and processing the data, it turned out that the screening laboratories were in many cases so familiar with the coding structure set forth in Instruction 14 that they found it a nuisance to write the natural language for these fields. The option of encoding was then added. The program recognizes this option by the asterisk (*) preceding the code. The shaded blocks on the forms represent the areas where the codes are to be placed.

Another change included additional room for control animals. Combination chemotherapy control packs tend to be larger than primary screening packs and, according to the screening protocol (1), more animals are required. Since there are ten fields that may vary in an experiment and since each of these fields has many alternatives, the possibilities within a control pack are astronomical. Although the number of tests involved is larger (generally speaking) in combination chemotherapy packs, the number of materials or therapies involved is smaller because other variations are being introduced into the experiment. As a result, the area of the form devoted to NSC numbers and number of tests within each NSC number or combination of NSC numbers was made more compact and a separate area for vehicle tests was introduced. In this form, there is no area for the number of tests in each type of material (required on the single-therapy form NIH-1157) because most of the materials used have been assigned synthetic NSC numbers and, as mentioned earlier, they may not be, even strictly speaking, materials. The other major difference between this form and form NIH-1157 is that laboratories are requested to record deaths rather than survivors.

The biggest change from the single-therapy forms was the introduction of what is called the Screening Test Element (*see* fig 2, form NIH-1744-2). This new form is what allows the variations in the testing that were discussed earlier to be entered into the system with minimum effort. One form NIH-1744-2 must be completed for each change in the variable fields listed previously (with the exception of dose levels). Vehicle, route, and regimen fields may be completed in either natural language or by the appropriate codes (some of which are printed on the form). Each page is assigned a combination number (card columns 7 and 8) by the screening laboratory. This is a sequential number starting with one (1) and is for identifying the descriptors of the individual tests.

The Screening Test Record (*see* fig 3, form NIH-1744-3) is a product of the information recorded on the screening test element. Only the primary descriptors (screener number-control number) need be entered. A complete description of the variable fields is inherent in the two numbers entered for each portion of the test—combination number and dose number. If only one material is used, only one combination-number/dose-number pair is completed on the form. When the order of therapy administration is significant, the combination-number/dose-number pair should be entered accordingly. Also, there is enough room in the regimen field for comments such as "administered 15 minutes prior to NSC-00000."

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Public Health Service
National Institutes of Health
National Cancer Institute
Drug Research and Development
Bethesda, Maryland 20014

COMBINATION CHEMOTHERAPY SCREENING CONTROL RECORD

SCREENER	CONTROL NUMBER	HOST	TUMOR	PARAM.	INOCULUM				DATE ON			BODY WT. CHANGE	AV. EVALUATION
					SITE	TIS.	LEVEL	% TUMOR	YEAR	MONTH	DAY		
1 2	3 4 5 6	7 8	9 10	11	12	13	14	15 16	17 18	19 20	21 22	23 24 25 26	27 28 29 30

VEHICLE																ROUTE										
23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51

BACT.		TUMOR SUBLINE			ANIMALS				FINAL EVAL. DAY		NO. TAKES		POSITIVE CONTROL										NEGATIVE			C. S. C.
1	2	LINE	CODE	GEN.	SEX	SOURCE		NUMBER					NSC NUMBER						% T/C		NO. SURVIVORS		% T/C			
		54	55	56	57	58	59	60	61	62	63	64	65	66	67	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	78

DEATHS

DAY																										
NO.																										

NO.	ANIMAL WEIGHTS INITIAL DAY		ANIMAL WEIGHTS TOXICITY EVALUATION DAY		TUMOR EVALUATION				NO.	ANIMAL WEIGHTS INITIAL DAY		ANIMAL WEIGHTS TOXICITY EVALUATION DAY		TUMOR EVALUATION				NSC NUMBERS	NUMBER OF TESTS	
	68	69	70	71																	
1									25												
2									26												
3									27												
4									28												
5									29												
6									30												
7									31												
8									32												
9									33												
10									34												
11									35												
12									36												
13									37										TOTAL DRUG TESTS	72	73
14									38												
15									39										VEHICLE TESTS	74	75
16									40												
17									41										GRAND TOTAL ALL TESTS	76	77
18									42										COMMENTS		
19									43												
20									44												
21									45												
22									46												
23									47												
24									48												
									49												

NIH-1744-1
Rev. 10-73

DPC/DR&D

FIGURE 1.—Combination Chemotherapy Screening Control Record (form NIH-1744-1).

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National Cancer Institute
Drug Research and Development
Bethesda, Maryland 20014

COMBINATION CHEMOTHERAPY SCREENING TEST ELEMENT

SCREENER		CONTROL NUMBER				COMB. NUMBER		HOST		INOC. SITE	INOC. LEVEL	NSC NUMBER PRE	NSC NUMBER CORE						NSC NUMBER SAMPLE NO.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23

VEHICLE																ROUTE								
24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50

REGIMEN																						DAY 1ST INJ.		NO. OF INJECTIONS		SCHEDULE
51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79

DOSE NUMBER		DOSE/INJECTION								
0	1									
0	2									
0	3									
0	4									
0	5									
0	6									
0	7									
0	8									
0	9									
1	0									
1	1									
1	2									
1	3									
1	4									
1	5									

CODES

VEHICLE

0 - None
1 - Methylcellulose (MC)
2 - Saline
3 - Acid diluted with saline
4 - Steroid suspending solution
5 - Alkali diluted with saline
6 - Olive oil, sesame oil, peanut oil
7 - Other
8 - Carboxymethylcellulose (CMC)
9 - Water
A - Normal Media
B - Propylene Glycol
C - Acetone
D - Alcohol
E - Dimethyl Formamide
F - Dioxane
G - Dextrose
H - Acid diluted with CMC
K - Lactate
L - Clinical formulation
M - Klucel (Hydroxypropylcellulose) (HPC)
P - PVP
Q - Citric Acid
R - Lactic Acid
S - Saline Sonified
T - Saline with Tween-80
U - Gum Acacia
V - Sodium Bicarbonate
W - Saline + Tween 80 + Alkali
X - DMSO
Y - Alkali diluted with CMC
Z - Saline + Alcohol

ROUTE

0 - None (Controls Only)
1 - IP (Intraperitoneal)
2 - SC (Subcutaneous)
3 - Oral
4 - Other
5 - IV (Intravenous)
6 - IM (Intramuscular)
7 - Oral with prior fast

DOSE UNITS

Dose per injection
M - mg/kg
G - Grams/kg
N - 1/1000 micrograms/kg
% - % of drug inhaled or (by weight) in feed
U - International units/animal

COMMENTS

DISTRIBUTION: WHITE - DPC/DR&D.
CANARY - Screener
MANILLA - Extra Copy

NIH - 1744-2
(Rev. 12-72)

FIGURE 2.—Combination Chemotherapy Screening Test Element (form NIH-1744-2).

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Public Health Service
National Institutes of Health
National Cancer Institute
Drug Research and Development
Bethesda, Maryland 20014

COMBINATION CHEMOTHERAPY

SCREENING TEST RECORD

SCREENER		CONTROL NUMBER				TEST NUMBER		NUMBER OF ANIMALS		NUMBER OF NO-TAKES		NUMBER OF CURES		STATUS CODE TEST		STATUS CODE SUF	CONT. STAT CODE
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER		COMB. NUMBER		DOSE NUMBER	
18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45

DEATHS

DAY																												
NO.																												

NO.	ANIMAL WEIGHTS INITIAL (DAY_____)			TOXICITY EVALUATION (DAY_____)			TUMOR EVALUATION				COMMENTS
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
TOT.											
AV.				X			X				

NIH-1744-3
(Rev. 3-72)

BODY WT. CHANGE (TOX. – INIT. DAY)						EVALUATION					% T/C THIS TEST			
±														

DPC/DR&D
Pg. 1

FIGURE 3.—Combination Chemotherapy Screening Test Record (form NIH-1744-3).

The use of alternatives to this method of data capture is under continual investigation. Small changes providing for greater ease in entering and editing data have already been introduced. Although this system is somewhat cumbersome, it has proved so far to be the most satisfactory.

STORING THE DATA

Compounds involved in combination testing are of great interest to the chemotherapy program. Since a completely separate file is maintained for this data, more detail is stored on it than on the single-compound-oriented files. Not only are all of the data descriptors stored, but individual death patterns are maintained as well. Soon to be added are individual tumor weights for future evaluation. The natural language that was mentioned earlier is also stored on the master files.

Quite aside from the storage and retrieval of data on this special file, a way had to be devised to integrate this data into the statistics and management reports of the rest of the data processing system. No simple solution to this problem was forthcoming and a series of Rube Goldberg measures had to be taken. First, those tests that comprise the portion of the experiment in which the animals receive one drug only are prepared for input to the main system with a special study code of "C," which indicates that they are a part of a combination study. Then, the doses are converted to the dose-response scheme and the natural language is translated to the coding system required by the main file. The control packs are then included in the microfilm index with an annotation as to how many of the tests have been stored on the single-compound-oriented files out of how many were received in total. The control lines are entered into the control line file so that quality control, management, and statistical reports will include this testing.

REPORTS

The primary reason for maintaining any file of scientific data is for future reference, evaluation, and comparison. This file is no exception. Although we have been receiving and processing data for some time, the system has been in such a state of growth that there has been little time to develop a variety of outputs. The integration of this system with the main system provides normal output for the single-drug tests, and data are reported in Screening Data Summaries (fig 4), Screening Experimental Analysis (SEA) Listings (fig 5), and the various management reports mentioned previously. In addition, two reports are produced from this separate system. One report, the Activity report, gives the highlights of each experiment. The reports on individual experiments are sent to the screener involved and to the Drug Evaluation Branch. The other report, the Combination Chemotherapy Cross-Reference Report, has a wider distribution since it serves as an index to experiments that have been processed by the computer programs and are available on the computer files for querying.

Combination Chemotherapy Tumored-Animal Activity Report

Figure 6 is a sample activity report where cyclophosphamide (NSC-26271), cytosine arabinoside (NSC-63878), and procarbazine (NSC-77213) have been combined. At the top of the page is the date that the test was started at the screening laboratory (test date) and the date that the report was printed and the information stored on the combination chemotherapy master file (report date). To the right, under the page number, is a list of the drugs involved in this test.

Control information is listed as follows across the top of the report: screener number, control number, evaluation of the control group (in this case, mean survival time), test system, and inoculum tissue and level. The next lines itemize the NSC number, sample number, and vehicle for each combination (test-element record) in the experiment. For this particular test, the evaluation of toxicity was made on Day 5 and the final evaluation was made on Day 60, as can be seen in the headings. The body of the report itemizes the various test parameters and the results of the testing.

DISTR. CODE: 140Q | 1 ST SCR: XX

SCREENING DATA SUMMARY

DRUG RESEARCH AND DEVELOPMENT
CHEMOTHERAPY
NATIONAL CANCER INSTITUTE BETHESDA, MD. 20014

NSC 26271
Date 12/13/72 Page 35

S.I. | Q.N.S.: | K
M.C.: P | DATE:
R.C.DATE:
PUB: 1 | COR: | OTHER: ST

SAMPLE NUMBER	HOST	TUMOR	PARAMETER	SITE	TISSUE	LEVEL	SCREENER	CONTROL NUMBER	DATE YR.	DATE MO.	VEHICLE	ROUTE	INTERVAL M	INTERVAL D	DAY 1ST INJ	NO. OF INJECTIONS	DAY OF EVALUATION	DOSE NO.	DOSE INJ.	TOXICITY DAY	TOXICITY SURVIVORS	CURES	CONTROL BODY WEIGHT CHANGE	ANIMAL WEIGHT DIFF. (T-C)	TUMOR EVALUATION TEST	TUMOR EVALUATION CONTROL	PERCENT (T/C) %	TEST STATUS CODE	C S C	M C
ML00	02	LE	2	1	15		02	4614	70	9	21		+1		1	01	60	1	200	7	7	4	.1	-2.8	44.1	10.2	432	24	1	C
ML00	02	LE	2	1	15		02	4614	70	9	21		+1		1	01	60	2	100	7	7		.1	-1.4	15.1	10.2	148	24	1	C
ML00	02	LE	2	1	15		02	4614	70	9	21		+1		1	01	60	3	50.0	7	7		.1	- .9	11.7	10.2	114	24	1	C
M902	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	1	800	1	6		.9	-6.1	6.0	9.3		24	1	
M902	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	2	400	5	6		.9	-2.2	31.0	9.3	333	24	1	
M902	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	3	200	6	6	4	.9	-2.0	25.8	9.3	277	24	1	
M902	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	4	100	6	6		.9	-2.1	13.7	9.3	147	24	1	
M905	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	1	800	0	6		.9	- .9	0.0	9.3		24	1	
M905	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	2	400	5	6	4	.9	-4.3	27.0	9.3	290	24	1	
M905	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	3	200	6	6	2	.9	-2.7	23.5	9.3	252	24	1	
M905	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	4	100	6	6	3	.9	-1.8	21.8	9.3	234	24	1	
M908	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	1	800	1	6		.9	-4.0	7.0	9.3		24	1	
M908	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	2	400	5	6	5	.9	-3.2	31.0	9.3	333	24	1	
M908	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	3	200	6	6	1	.9	-2.1	20.3	9.3	218	24	1	
M908	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	4	100	6	6		.9	-1.7	14.5	9.3	155	24	1	
M911	02	LE	2	1	15		02	4744	70	A	21		+1		1	01	30	1	800	2	6		.9	-4.0	7.0	9.3		24	1	

NIH 1166-1 (REV. 7/72)

A

Criteria for Activity

Tumors evaluated on the basis of survival (parameters 2, 3, or 4)

In general an increase in survival of Treated animals (compared to Control animals) resulting in a T/C% of 125 or more is required for further testing.

Tumors evaluated on the basis of tumor inhibition (parameters 1, 8, 9, A)

In general inhibition of tumor growth of Treated animals (as compared to Control animals) resulting in a T/C% of 42 or less is required for further testing.

In vitro testing is used primarily for bioassay related to fractionation of in vivo active natural products. Normally, materials are not developed further on the basis of in vitro activity alone. Currently, however, new natural products with an ED_{50} of 20 μg/ml, and synthetic materials with an ED_{50} of 4 μg/ml, are evaluated for further testing.

In subsequent testing, activities substantially greater than the minimal end joints described above are required (e.g., a T/C% of 150 in 3LE testing). Criteria for acceptance as a candidate for development toward clinical trial are dependent on a number of factors, including the predictive value and overall sensitivity of the tumor, as well as on the nature and physical-chemical properties of the test material. These criteria are reviewed periodically by the Chemotherapy Staff, NCI and are flexible. Suppliers of active materials will be notified of the progress of their materials by letter.

Note: Instruction 14, which defines the codes on this computer printout, is no longer being distributed routinely with the Screening Data Summaries.

B

FIGURE 4.—Screening Data Summary (sample). *A*. Front. *B*. Back.

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE, NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE, DRUG RESEARCH AND DEVELOPMENT
BETHESDA, MARYLAND, 20014

SCREENING EXPERIMENT ANALYSIS DATE 01/04/71 PAGE 1

HOST	TUMOR	SCREENER	CONTROL NUMBER	PARAMETER	INOCULUM SITE	INOCULUM TISSUE	INOCULUM LEVEL % TUMOR	DATE ON YEAR	DATE ON MO	DATE ON DAY	VEHICLE	ROUTE	NUMBER OF MATERIALS	NUMBER OF TESTS TOTAL	NUMBER OF TESTS SYNTH.	NUMBER OF TESTS PLANTS	NUMBER OF TESTS FERM. PRODS.	NUMBER OF TESTS ANIM. PRODS.	TUMOR SUBLINE LINE	TUMOR SUBLINE CODE	TUMOR SUBLINE GEN.	ANIMALS SEX	ANIMALS SOURCE	ANIMALS NUMBER	DAY TOXIC	DAY FINAL	DEATHS	NO TAKES	NEGATIVE CONTROL % T/C	n p n	SPEC. SITY.	POSITIVE CONTROL NSC NUMBER	STANDARD DEVIATION	BODY WEIGHT CHANGE	AVERAGE EVALUATION
02	LE	02	4614	2	1	1	599	70	9	02	2	1	03	09	09	00	00	00	22	R	11	M	11	300	05	60	00	00	00	01	1	C		0019	+001	0102

PREFIX	NSC NUMBER CORE	NSC NUMBER SAMPLE NUMBER	DISTRIBUTION CODE	VEHICLE	ROUTE	X INTERVAL	LO	DAY 1ST INJ	NUMBER OF INJECTIONS	STRONG VEH.	DOSE/INJ	TOXICITY DAY SURVIVORS ( ) OF ( )	CURES	NO TAKES	ANIMAL WEIGHT DIFF. (T-C)	AVERAGE TEST EVAL.	PERCENT (T/C) %	TEST STATUS CODE	ACTIVE TEST	SCREENER COMMENTS
	026271	ML00	*****	2	1	+1	1	01	1		200	7 7	4		-2.8	44.1	432	24	*	
	026271	ML00	140I	2	1	+1	1	01	2		100	7 7	0		-1.4	15.1	148	24	*	
	026271	ML00	*****	2	1	+1	1	01	3		50.0	7 7	0		- .9	11.7	114	24		
	063878	PA00	*****	2	1	3	4	1	24	1	96.0	4 7	0		-5.9	7.8		22		
	063878	PA00	035D	2	1	3	4	1	24	2	48.0	7 7	0		-2.7	12.0	117	22		
	063878	PA00	*****	2	1	3	4	1	24	3	24.0	7 7	3		- .3	41.3	404	22	*	
	077213	MD00	217A	2	1	+1	1	09	1		400	7 7	0		-6.5	11.7	114	22		
	077213	MD00	*****	2	1	+1	1	09	2		200	7 7	0		-5.1	16.0	156	22	*	
	077213	MD00	217A	2	1	+1	1	09	3		100	7 7	0		-3.7	13.6	133	22	*	

*9

5 ACTIVE TESTS WITH PARM/SITE OF 21 AND % T/C = OR > 125

FIGURE 5.—Screening Experimental Analysis Listing (sample).

DRUG RESEARCH & DEVELOPMENT, NCI
COMBINATION CHEMOTHERAPY ACTIVITY REPORT
TEST DATE - 09/23/70
REPORT DATE - 12/15/71

NSC
026271
063878
077213

SCREENER - 02 CONTROL - 4614 MEAN SURVIVAL TIME - 10.18 TEST SYSTEM - LE21 TISSUE/LEVEL - 15

COMB NO	NSC	SAMPLE	VEHICLE	COMB NO	NSC	SAMPLE	VEHICLE
1	26271	ML00	SALINE	3	77213	MD00	SALINE
2	63878	PA00	SALINE				

TEST NO	COMB NO	REGIMEN	INJECTIONS INIT	DAY NO	ROUTE	NSC NUMBER	DOSE/ INJECTION		WT CHNG DAY 5	MEAN SURVIVAL	% T/C	DAY 60 SURVIVORS
1	1	DAY 1 ONLY	1	1	IP	26271	200.0	MG/KG	-2.63	43.56	427	04/07
2	1	DAY 1 ONLY	1	1	IP	26271	100.0	MG/KG	-1.34	15.13	148	00/07
3	1	DAY 1 ONLY	1	1	IP	26271	50.0	MG/KG	-0.78	11.69	114	00/07
4	2	Q3H DAYS 1,5,9	1	24	IP	63878	96.0	MG/KG	-5.81	7.75	76	00/07
5	2	Q3H DAYS 1,5,9	1	24	IP	63878	48.0	MG/KG	-2.63	12.00	117	00/07
6	2	Q3H DAYS 1,5,9	1	24	IP	63878	24.0	MG/KG	-0.28	41.00	402	03/07
7	3	DAYS 1-9	1	9	IP	77213	400.0	MG/KG	-6.41	11.69	114	00/07
8	3	DAYS 1-9	1	9	IP	77213	200.0	MG/KG	-4.91	16.00	157	00/07
9	3	DAYS 1-9	1	9	IP	77213	100.0	MG/KG	-3.56	13.56	133	00/07
10	1	DAY 1 ONLY	1	1	IP	26271	100.0	MG/KG	-5.63	9.56	93	00/07
	3	DAYS 1-9	1	9	IP	77213	200.0	MG/KG				
11	1	DAY 1 ONLY	1	1	IP	26271	50.0	MG/KG	-4.00	17.69	173	00/07
	3	DAYS 1-9	1	9	IP	77213	100.0	MG/KG				
12	1	DAY 1 ONLY	1	1	IP	26271	25.0	MG/KG	-2.19	14.28	140	00/07
	3	DAYS 1-9	1	9	IP	77213	50.0	MG/KG				
13	2	Q3H DAYS 1,5,9	1	24	IP	63878	48.0	MG/KG	-6.13	7.84	76	00/07
	3	DAYS 1-9	1	9	IP	77213	200.0	MG/KG				
14	2	Q3H DAYS 1,5,9	1	24	IP	63878	24.0	MG/KG	-5.34	9.56	93	00/07
	3	DAYS 1-9	1	9	IP	77213	100.0	MG/KG				

FIGURE 6.—Combination Chemotherapy Tumored-Animal Activity Report (sample).

Combination Chemotherapy Toxicity-Activity Report

Figure 7 shows a sample of a nontumored-animal toxicity report. It is similar in format to the tumored-animal activity report with the exception that there are control identifiers only in the heading and if the test was run in connection with a tumored-animal test the control number of that test is noted on the right side of the top line of the report.

The weights listed for each test are for the test animals only. This is in contrast to the tumored-animal report in which the mean weight change of each test group minus the mean weight change of the control group is listed.

Combination Chemotherapy Cross-Reference Report

In figure 8, a typical page of the cross-reference report is shown. The chemical name of the compound designated "NSC-1" has recently been added. On this sample page, tests combining actinomycin D (NSC-3053) with emetine hydrochloride (NSC-33669), vincristine (NSC-67574), and L-asparaginase (NSC-109229) and tests combining 6-MP riboside (NSC-4911), mephalan (NSC-8806), prednisone (NSC-10023), and 5-FU (NSC-19893) with other drugs are shown.[2] Note that two NSC numbers—NSC-3069 and NSC-15200—have no chemical name assigned to them. The name file is maintained separately from the cross-reference file. As new NSC numbers appear on the cross-reference report, the chemical name file is updated with names approved by the Drug Development Branch. Since this report is always cumulative, the missing names will appear in later versions as they are added to the file.

A cross-reference report is prepared whenever the combination chemotherapy master file is updated. Normally this file is updated on a biweekly schedule, which is an alternative schedule to the one for the updating of the main file. During this period when we are trying to place a large backlog of data received onto the file, the file will be updated more often—perhaps as often as two or three times a week.

The cross-reference report is organized as if all of the testing were done with two-drug combinations. This format is subject to modification or to the introduction of alternatives in the future. For now, it is necessary to find combinations of more than two drugs by comparing screener, test system, and control number. Figure 9 is a sample page of the cross-reference report showing ICRF-159 (NSC-129943) combined with emetine hydrochloride (NSC-33669) and vinblastine (NSC-49842) in test system 3LE31, control number 1949, at screener 01.

When a control number, screener, and compound combination appears twice, it is because the test was evaluated by two separate parameters. For example, figure 8 shows screener 01, control number 0159, combining prednisone (NSC–10023) and bleomycin (NSC–125066) evaluated by parameter 3 (median survival time) and parameter 8 (mean tumor weight).

COMMENT

As mentioned earlier in this paper, the programs, methods of retrieval and data capture, and the format and types of reports available from this system are still under evaluation. As the data base grows, needs and new approaches to handling the data will become obvious and they will be incorporated into the system.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

Bleomycin: NSC-125066

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C

Emetine hydrochloride: NSC-33669; CAS reg. No. 316-42-7

5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4′-propylenedi-, (±)-

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

DRUG RESEARCH & DEVELOPMENT, NCI
COMBINATION CHEMOTHERAPY ACTIVITY REPORT
TEST DATE - 11/18/70
REPORT DATE - 10/26/71

PAGE 1

NSC
026271
119875

SCREENER - 01 CONTROL - 0236 AA TOXICITY TEST TUMORED CONTROL - 0119

COMB NO	NSC	SAMPLE	VEHICLE	COMB NO	NSC	SAMPLE	VEHICLE
1	26271	ML00	SALINE	2	119875	ML00	SALINE

TEST NO	COMB NO	REGIMEN	INJECTIONS INIT	DAY NO	ROUTE	NSC NUMBER	DOSE/ INJECTION		WT CHNG DAY 5	MEDIAN SURVIVAL	% T/C	DAY 59 SURVIVORS
1	1	DAY 1 ONLY	1	1	IP	26271	500.0	MG/KG	-4.47	7.00		00/08
2	1	DAY 1 ONLY	1	1	IP	26271	300.0	MG/KG	-1.88	33.00		05/08
3	1	DAY 1 ONLY	1	1	IP	26271	180.0	MG/KG	-1.25	33.00		05/08
4	2	DAY 1 ONLY	1	1	IP	119875	14.0	MG/KG	-4.88	7.50		01/08
5	2	DAY 1 ONLY	1	1	IP	119875	8.4	MG/KG	-2.50	59.00		08/08
6	2	DAY 1 ONLY	1	1	IP	119875	5.0	MG/KG	-1.50	59.00		08/08
7	1	DAY 1 ONLY	1	1	IP	26271	300.0	MG/KG	-3.13	4.00		01/08
	2	DAY 1 ONLY	1	1	IP	119875	8.4	MG/KG				
8	1	DAY 1 ONLY	1	1	IP	26271	300.0	MG/KG	-4.94	17.00		01/08
	2	DAY 1 ONLY	1	1	IP	119875	5.0	MG/KG				
9	1	DAY 1 ONLY	1	1	IP	26271	300.0	MG/KG	-2.91	34.00		06/08
	2	DAY 1 ONLY	1	1	IP	119875	3.0	MG/KG				
10	1	DAY 1 ONLY	1	1	IP	26271	180.0	MG/KG	-4.63	34.00		06/08
	2	DAY 1 ONLY	1	1	IP	119875	8.4	MG/KG				
11	1	DAY 1 ONLY	1	1	IP	26271	180.0	MG/KG	-2.75	34.00		07/08
	2	DAY 1 ONLY	1	1	IP	119875	5.0	MG/KG				
12	1	DAY 1 ONLY	1	1	IP	26271	180.0	MG/KG	-1.25	34.00		07/08
	2	DAY 1 ONLY	1	1	IP	119875	3.0	MG/KG				

FIGURE 7.—Combination Chemotherapy Toxicity-Activity Report (sample).

DRUG RESEARCH & DEVELOPMENT,DCT,NCI

COMBINATION CHEMOTHERAPY

COMBINATION CROSS-REFERENCE

REPORT DATE 02/15/73

CHEMICAL NAME	NSC 1	NSC 2	SCREENER	CONTROL	TEST SYSTEM
ACTINOMYCIN D	3053	33669	40	0103	3LE21
ACTINOMYCIN D	3053	67574	01	1651	3LE31
ACTINOMYCIN D	3053	109229	40	0053	3LE21
	3069	26271	01	0291	3AA3
6-MP RIBOSIDE	4911	33669	40	0040	3LE21
MELPHALAN	8806	79037	08	C415	3LE29
PREDNISONE	10023	26271	01	0047	3LL36
			01	0250	3AA3
			01	1963	3LE31
PREDNISONE	10023	67574	01	0708	3LE32
			40	0019	3AK31
PREDNISONE	10023	125066	01	0159	3LL16
			01	0159	3LL36
PREDNISONE	10023	135758	40	0144	3LE21
	15200	107392	01	2100	3LE31
5-FU	19893	740	40	0036	3LE21

*NOTE: 3AKF3 TEST RESULTS ARE NOT RESIDENT ON THE COMBINATION CHEMOTHERAPY FILES AT NCI

FIGURE 8.—Combination Chemotherapy Cross-Reference Report (sample). (A cross reference to the combination chemotherapy testing results stored on a file at Southern Research Institute [SRI] has been added to our report in cooperation with Dr. Harris Lloyd. The data have not been moved to the NIH files. The note at the bottom of the page serves as a notice that information on this testing would have to be requested from SRI.)

DRUG RESEARCH & DEVELOPMENT, DCT, NCI

COMBINATION CHEMOTHERAPY

COMBINATION CROSS-REFERENCE

REPORT DATE 07/16/73

CHEMICAL NAME	NSC 1	NSC 2	SCREENER	CONTROL	TEST SYSTEM
ICRF-159	129943	26271	01	0134	3LL16
			01	0134	3LL36
			01	0316	3AA3
			01	1994	3LE32
ICRF-159	129943	33669	01	1949	3LE31
			01	1974	3LE31
ICRF-159	129943	49842	01	1949	3LE31
			01	1974	3LE31
ICRF-159	129943	58514	01	2089	3LE31
ICRF-159	129943	63878	01	1951	3LE27
			01	1994	3LE32
ICRF-159	129943	71795	01	2091	3LE31
			40	0098	3LE21
			40	0114	3LE21
ICRF-159	129943	82151	01	0001	3LE29
			01	0254	3AA3
			01	0257	3AA3
			01	0261	3AA31
			01	0275	3AA3
			01	0301	3AA3
			01	0311	3AA3
			01	0313	3AA3
			01	0320	3AA2
			01	2003	3LE31
			01	2015	3LE31
			01	2033	3LE31
			01	2068	3LE31
			01	2082	3LE31
			01	2104	3LE31

*NOTE: 3AKF3 TEST RESULTS ARE NOT RESIDENT ON THE COMBINATION CHEMOTHERAPY FILES AT NCI

FIGURE 9.—Combination Chemotherapy Cross-Reference Report (sample). (*See* legend for figure 8.)

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

6-MP riboside: NSC-4911; CAS reg. No. 574-25-4; mercaptopurine riboside; 9*H*-purine-6-thiol, 9-β-D-ribofuranosyl-

NSC-740: methotrexate; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl-; CAS reg. No. 59-05-2

NSC-3069: chloramphenicol; acetamide, 2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]-; CAS reg. No. 56-75-7

NSC-15200: gallium nitrate; CAS reg. No. 13494-90-1

NSC-58514: chromomycin A_3; CAS reg. No. 7059-24-7

NSC-71795: ellipticine; 6*H*-pyrido[4,3-*b*]carbazole, 5,11-dimethyl-; CAS reg. No. 519-23-3

NSC-79037: urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-; CCNU; CAS reg. No. 13010-47-4

NSC-82151: daunomycin; daunorubicin (NSC-83142); CAS reg. No. 23541-50-6

NSC-107392: 5-hydroxypicolinaldehyde thiosemicarbazone; 5-HP; CAS reg. No. 19494-89-4

NSC-119875: *cis*-diamminedichloroplatinum; CAS reg. No. 15663-27-1

NSC-135758: 2,5-piperazinedione, 3,6-bis(5-chloro-2-piperidyl)-, dihydrochloride

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

REFERENCE

1. GERAN RI, GREENBERG NH, MACDONALD MM, ET AL. Protocols for screening chemical agents and natural products against animal tumors and other biological systems (3d edition). Cancer Chemother Rep Part 3, vol 3:1–103, 1972.

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